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*Virginia Commonwealth University*

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THE ROLE OF CANNABINOIDS AND CANNABINOID RECEPTORS IN ENTERIC  
NEURONAL SURVIVAL

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

by

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## List of Abbreviations

2-AG	2-arachidonoylglycerol
5-HT <sub>3</sub>	5-hydroxytryptamine 3 receptor
ACEA	Arachidonyl-2'-chloroethylamide
Ach	Acetylcholine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AEA	Anandamide
AraC	Arabinoside cytosine
Anadamide	Arachidonylethanolamide
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
CGRP	Calcitonin Gene-Related Peptide
ChAT	Choline Acetyltransferase
CNS	Central nervous system
COX	Cyclooxygenase
CRD	Colorectal distension
D2 receptor	Dopamine D2 receptor

DAB	3, 3' Diaminobenzidine
DMEM	Dulbecco Modified Eagle's Medium
DNBS	2,4-dinitrobenzene sulfonic acid
DNS	Donkey normal serum
DSS	Dextran sulfate sodium
EMT	Endocannabinoid membrane transporter
eNOS	Endothelial Nitric Oxide Synthase
ENS	Enteric nervous system
FAAH	Fatty acid amide hydrolase
FBS	Fetal bovine serum
FEPPSPs	Fast excitatory postsynaptic potentials
GDNF	Glial-derived neurotrophic factor
GI	Gastrointestinal tract
GIRK1/4	G protein-coupled inwardly rectifying potassium1/4
GPCR	G protein coupled receptor
HD	Huntington's disease
HRP	Horseradish peroxidase
HU-210	11-hydroxy- $\Delta^8$ -THC-dimethylheptyl
IBD	Inflammatory bowel disease
ICC-DMP	Deep muscular interstitial cells of Cajal
ICC-IM	Intramuscular interstitial cells of Cajal
ICC	Interstitial cells of Cajal

ICC-MY	Myenteric interstitial cells of Cajal
ICC-SM	Submucosal interstitial cells of Cajal
IFN $\gamma$	Interferon gamma
IJP	Inhibitory junctional potential
IL-12	Interleukin-12
IL-1 $\beta$	Interleukin-1 $\beta$
IL-23	Interleukin-23
IL-6	Interleukin-6
IM-FEN	Immorto fetal enteric neuronal cell line
IPANs	Intrinsic primary afferent neurons
LPS	Lipopolysaccharides
MAGL	Monoacylglycerol lipase
MAPK	mitogen activated protein kinase
MEK	MAP kinase kinase
MGluR	Metabotropic glutamate receptor
MOR1	mu-opioid receptor
MPO	Myeloperoxidase
MPOs	Myenteric potential oscillations
MS	Multiple sclerosis
NA	Nucleus accumbens
NAPE	Phosphatidylethanolamine
NAT	N-acyl transferase
NGF	Nerve growth factor

NGS	Normal goat serum
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
OM	Oil of mustard
PACAP	Pituitary adenylate cyclase activating polypeptide
p-AKT	Phospho-AKT
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEA	Palmitoylethanolamide
PGP9.5	Protein gene product 9.5
PI-3K	Phosphatidylinositol-3-kinase
PKB	Protein Kinase B
PLC	Phospholipase C
PLC- $\beta$	Phospholipase C beta
PLD	Phospholipase D
p-P44/42MAPK	Phospho-P44/42 mitogen activated protein kinase
PTX	Pertussis toxin
SEM	Standard error of the mean
SP	Substance P
TBST	Tris Buffered Saline Tween-20

TNBS	Trinitrobenzene sulfonic acid
TNF- $\alpha$	Tumour necrosis factor-alpha
TRPV1	Transient receptor potential vanilloid subtype 1
VIP	Vasoactive intestinal peptide
$\Delta^9$ -THC	$\Delta^9$ -tetrahydrocannabinol



# **ABSTRACT**

## **THE ROLE OF CANNABINOIDS AND CANNABINOID RECEPTORS IN ENTERIC NEURONAL SURVIVAL**

By Yan Li

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University 2009

Major Director: John R. Grider,

Professor, Department of Physiology and Biophysics

The Endocannabinoid system has been found in the gastrointestinal tract, where it plays an important role in gut under both physiological and pathological conditions. Although the major effects of cannabinoids in the gut are mediated through effects on enteric neurons, the role of cannabinoids in the enteric nervous system is poorly understood. In the present study, we have used the primary cultures of myenteric ganglia and a newly developed fetal enteric neuronal cell line to identify whether the endocannabinoid, anandamide, affects ganglionic and neuronal survival and the pathways involved.

Anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentrations (1nM-0.1uM) and decreasing survival at high concentrations (1-10uM). Maximal survival (68% increase in number of ganglia surviving) occurred at 0.1uM and the ED50 was 3nM. This effect on promoting survival was inhibited by the CB1 antagonist AM251 (1uM) and by AraC (10uM), but not the CB2 antagonist AM630

(1uM). AM630 (1uM) significantly blocked the decreased survival induced by high concentration anandamide (10uM). The enteric glia was involved in anandamide-induced ganglion survival. Anandamide had no effect on the number of neurons/ganglion in the presence of enteric glia, but decreased the number of neurons/ganglion by 15-20% in absence of enteric glia. This effect was partially reversed by CB1 antagonist, AM251 (1uM) (20%-145% at 1nM-10uM) and by CB2 antagonist AM630 (1uM) (40%-185% at 1nM-10uM). In the fetal enteric neural cell line (IM-FEN), anandamide decreased enteric neuronal survival in a concentration-dependent manner at both 39 and 33 degree (11-45% and 10-22% decrease in survival at 1nM-10uM, respectively). Coculture of astrocytes with the enteric neuronal cells was not able to reverse anandamide-mediated neuronal death. Immunocytochemistry and western blot confirmed that the presence of both CB1 and CB2 receptors in enteric neurons (primary cultures and IM-FEN) and glia (primary cultures). In addition, the PLC-beta inhibitor U73122 (1uM) inhibited anandamide induced ganglia survival significantly. Anandamide also induced increased expression of phospho-P44/42MAPK (13-48% at 1nM-10uM) and phospho-AKT (1-28% at 1 nM-10uM) in IM-FEN.

We conclude that anandamide has a differential effect on survival of enteric ganglia and neurons. It promotes ganglionic and neuronal survival by CB1 receptors in the presence of glia and this involves the PLC-beta pathway. Conversely, anandamide promotes neuron death in absence of glia as a result of effects on both the MAPK and PI-3K/AKT pathways. Since the endocannabinoid system is upregulated in inflammatory bowel diseases, these effects may play a role in the pathogenesis of the response to inflammation as well as the recovery and reinnervation of the gut following the acute

phase of inflammation. The further significance of this work could contribute to developing new therapeutic methods for treatment of inflammatory bowel disease and related symptoms in clinic practice.

## **Introduction**

### **Enteric nervous system**

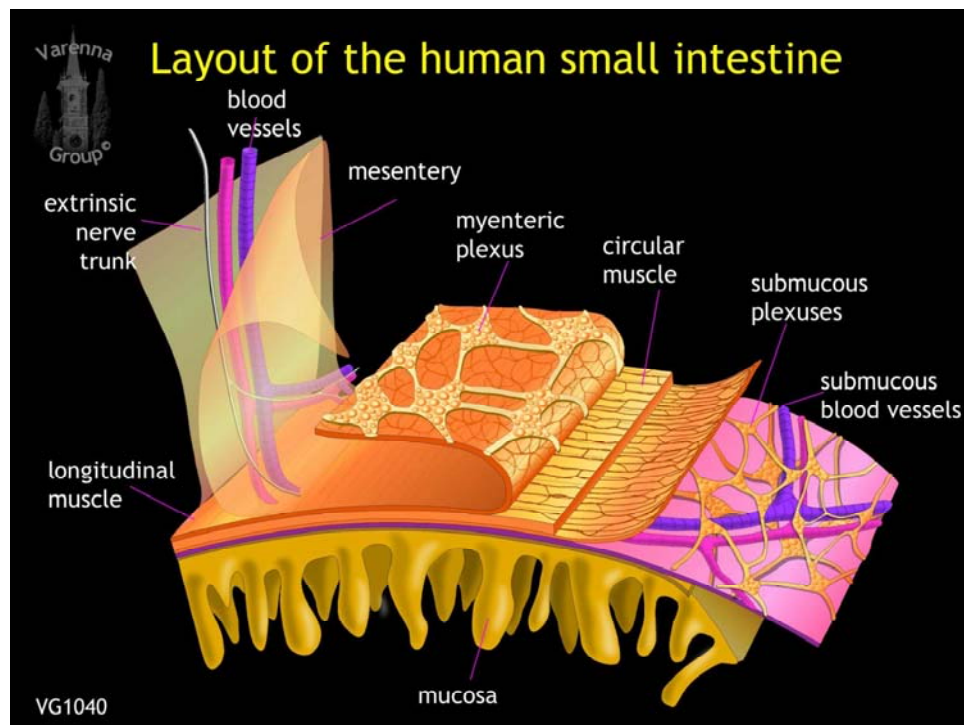
The gut is innervated by both extrinsic (parasympathetic and sympathetic) and intrinsic enteric nervous system (ENS). The extrinsic parasympathetic inputs originate in the dorsal motor nucleus of the vagus, which controls primarily the motility of the esophagus and stomach, and the sacral parasympathetic nucleus, which contributes to control of motility of the distal colon and rectum. The sympathetic adrenergic fibers from the prevertebral ganglia innervate the secretomotor neurons, presynaptic cholinergic nerve endings, submucosal blood vessels, and the sphincters of the GI tract.

The enteric nervous system consists of the ganglia which are grouped into two plexuses: the myenteric plexus (Auerbach's plexus), located between the outer longitudinal and the inner circular muscle layers, and submucosal plexus (Meissner plexus), located between the circular muscle and the muscularis mucosa (Figure 1). The myenteric plexus primarily provides motor innervation to the muscle layers and some secretomotor innervation to the mucosa. The submucosal plexus mainly regulates mucosal secretion and blood flow (1).

### *Enteric neurons*

The enteric ganglia consist of enteric neurons and glia. Enteric neurons are classified into different categories based on their histochemical, electrophysiologic and functional

Figure 1. Structure of the gut wall. The GI tract can be divided into: mucosa, submucosa, circular muscle layer, longitudinal muscle layer and serosa from inside to outside in order. Myenteric plexus (Auerbach's plexus) is located between the inner circular muscle layers and outer longitudinal, and submucosal plexus (Meissner plexus), located between the circular muscle and the muscularis mucosa. Adapted from John B. Furness<sup>136</sup>



properties. They are functionally classified into intrinsic primary afferent neurons (IPANs), interneurons, motor neurons, secretomotor neurons, and vasomotor neurons.

The IPANs are located in both the myenteric and submucosal plexuses. AH/Dogiel Type II neurons have smooth oval cell bodies with multiple processes (Dogiel Type II) and prolonged hyperpolarization after action potential, which have been found in the myenteric and submucosal plexuses of the small and large intestines. There is considerable evidence that AH neurons behave as intrinsic sensory neurons as they respond to a variety of chemical and mechanical stimuli applied to the mucosa and to muscle stretch and contraction (2-9). Local chemical and mechanical stimulation of the mucosa activate IPANs in part via release of serotonin from enterochromaffin cells, and then serotonin activate terminals of IPANs via 5-HT<sub>4</sub> receptors (10,11).

The other electrophysiological type of neurons are S/Type I neurons, which have a variety of cell shapes and are uniaxonal. Fast excitatory postsynaptic potential (FEPPSPs) can be readily evoked in S neurons. S neurons comprise the motor neurons and most of the interneurons. There are some mechanosensitive interneurons found in the guinea pig distal colon where they respond directly to changes in muscle length (circumferential stretch and longitudinal stretch), rather than muscle tone or tension (12).

The peristaltic reflex is a coordination of IPANs, interneurons and excitatory and inhibitory motoneurons which allows the normal propulsion of the contents of the gut.

The ascending excitatory reflex involves myenteric motor neurons that utilize Ach and substance P and elicit contraction of the smooth muscle located orally to the site of stimulation. The descending inhibitory reflex involves inhibitory motor neurons that utilize NO, VIP and PACAP and elicit relaxation of the smooth muscle located anally to

the site of stimulation (13).

### *Enteric glia*

The most abundant cells in the ENS are enteric glial cells that lie adjacent to the neurons in the enteric ganglia and envelop both their cell bodies and axon bundles (9). They were also found in the interconnecting nerve strands of the ganglionated and in all non-ganglionated plexuses, submucosal blood vessels and the mucosal epithelium (14-20).

The enteric glia has an important role in regulating barrier function of the intestinal epithelium (21-22) and neurochemical coding of enteric neurons (23). Proinflammatory cytokines induced neurotrophic factors glial-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) expression in enteric glia, suggesting their active role in inflammation (24-25). Recently, the communication between neuron and glia has been found in both guinea pig and mice where neuron released ATP could elicit a  $\text{Ca}^{2+}$  response in enteric glia (26-27). In addition, lipopolysaccharides (LPS) enhanced the action of bradykinin in enteric neurons via secretion of interleukin-1 $\beta$  (IL-1 $\beta$ ) from enteric glial cells in neonatal rats (28).

### **Interstitial cells of Cajal**

Interstitial cells of Cajal (ICC) are a non-neural cell type of similar mesenchymal origin to the muscle, which have been found throughout the gastrointestinal tract from the esophagus to the anus in a wide variety of species including humans. Morphological and physiological studies indicate that ICC play key roles in peristaltic movement as pacemaker cells and as mediators of neural activity to the gastrointestinal musculature



(29). Each part of the digestive tract shows a specific distribution of ICC.

ICC show a highly branched morphology and form unique networks including myenteric ICC at the level of the myenteric plexus (ICC-MY), intramuscular ICC in the musculature (ICC-IM), ICC in deep muscular plexus layer in the small intestine (ICC-DMP) and submucosal ICC at the submucosal surface of the circular muscle layer (ICC-SM). ICC-MY and ICC-SM serve as electrical pacemakers, generating slow waves, whereas ICC-IM and ICC-DMP are mediators of enteric motor neuron transmission so that neural influence is superimposed on the rhythmic activity of the muscle generated by ICC. ICC are primarily innervated by nitrergic (nNOS-containing) enteric neurons (30). However, inhibitory neurotransmitters can reach smooth muscle cells without hindrance when ICC are absent in fundus of stomach (31). In the small intestine, electrical slow waves are generated by ICC-MY, whereas slow waves in the colon originate in ICC-SM. The reason could be due to that ICC-SM form a tightly coupled network that is able to generate and propagate slow waves in the colon; in contrast,  $\text{Ca}^{2+}$  transients in ICC-MY which are normally not synchronized, have a similar duration and frequency as myenteric potential oscillations (MPOs). Like MPOs, their activity is inhibited by nitrergic nerves and synchronized by excitatory nerves (32).

## **Endocannabinoid system**

### *Endocannabinoids*

Cannabinoids have a long history of consumption for recreational and medical reasons.  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) is the primary active constituent of the hemp plant *Cannabis sativa*, which is mediated by at least two types of receptors (CB1 and CB2

receptors). Both of them are coupled to  $G_{i/o}$  proteins (33,34). The endogenous ligands for these receptors are also detected in mammalian tissues which are eicosanoids including arachidonylethanolamide (anandamide) and 2-arachidonoyl glycerol (2-AG) (35). Endocannabinoids are generated and released on demand after cellular depolarization in a calcium-dependent manner (36) or by activating dendritic metabotropic glutamate receptors (mGluRs) in a calcium-independent manner (37,38).

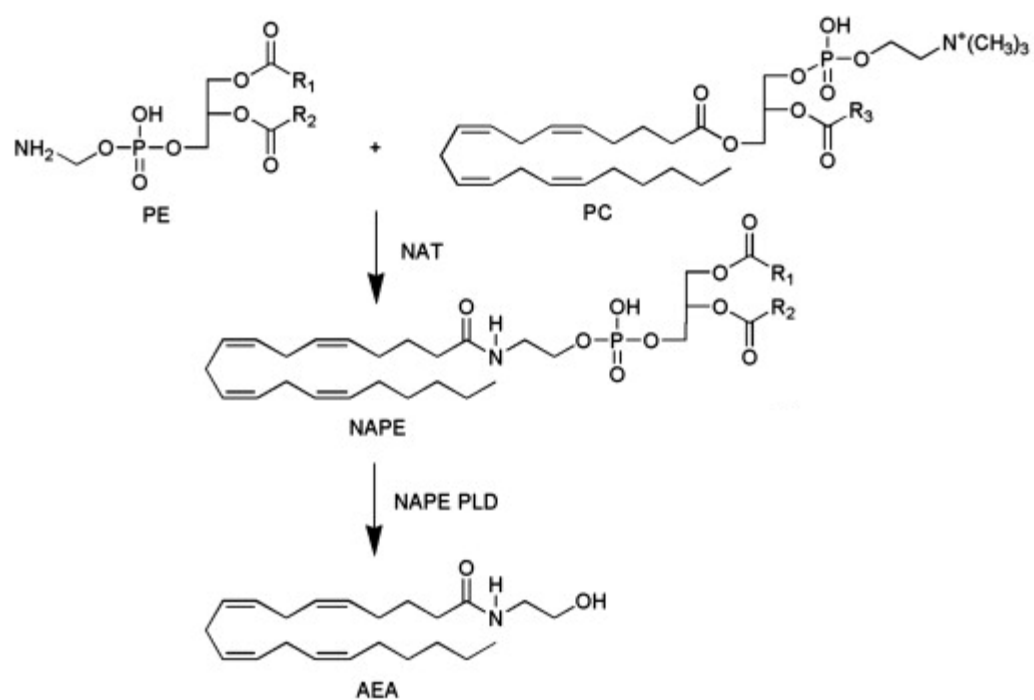
Different pathways are involved in the synthesis and release of anandamide and 2-AG. Anandamide is formed by the cleavage of a phosphatidylethanolamine (NAPE), which is catalysed by a specific phospholipase D (PLD) (39) (Figure2). 2-AG is mainly synthesized by the receptor-dependent activation of phosphatidylinositol-specific phospholipase C (PLC) (40,41). Once anandamide and 2-AG are formed, they target the CB1 receptors in the same cell where they were formed (42) or they can be released to the presynaptic terminals, acting as the retrograde synaptic messengers (43) in the brain. Endocannabinoids are removed from their sites of action by tissue uptake processes which could be involved in endocannabinoid membrane transporter (EMT) and metabolized mainly by fatty acid amide hydrolase (FAAH) for anandamide or monoacylglycerol lipase (MAGL) for 2-AG (35).

### *CB1 and CB2 receptors*

CB1 receptors are found predominantly at central and peripheral nerve terminals and expressed at high levels in the hippocampus, cortex, cerebellum, and basal ganglia (44-46). CB2 receptors occur mainly on immune cells, one of their roles being to modulate cytokine release (47). Recently GPR55, an orphan G protein-coupled receptor,

Figure 2. A schematic representation of the putative pathway for anandamide biosynthesis

N-acyl transferase (NAT), using phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as substrates, transfers a fatty acyl chain from the sn-1 position of a glycerolipid to PE in a calcium-dependent fashion, yielding N-arachidonyl phosphatidylethanolamine (NAPE). The formation of anandamide (AEA) is catalyzed by a calcium-dependent NAPE phospholipase D (NAPE PLD). Adopted from Ekaterina A. Placzek<sup>137</sup>



has been proposed as a new member of the cannabinoid receptors (48).

Endocannabinoids exhibit different binding properties and intrinsic activity at CB1 and CB2 receptors. Anandamide behaves as a partial agonist at both CB1 and CB2 receptors, but has higher affinity for the CB1 receptor (49,50). The intrinsic activity of anandamide at CB1 receptors is 4–30 fold higher than at CB2 receptors. However, 2-AG is a complete agonist at both CB1 and CB2 receptors and it exhibits less affinity than anandamide for both CB1 and CB2 receptors (40,49).

Taken together, endocannabinoids, their synthetic and degradative enzymes, eCB transporters, and cannabinoid receptors constitute the ‘endocannabinoid system’.

### **Pharmacology of cannabinoids**

The mainly used cannabinoid reagents are summarized in Table1 and the structure of typical cannabinoid agonists shown in Figure3.

#### *Cannabinoid receptor agonists*

According to the International Union of Pharmacology, cannabinoid agonists can be divided into classical cannabinoids, non-classical cannabinoids, aminoalkylindoles and eicosanoids. Classical cannabinoids are tricyclic dibenzopyran derivatives that are either compounds occurring naturally in the plant *C. sativa*, or synthetic analogues of these compounds. The most representative forms are  $\Delta^9$ -THC, a partial agonist at both the CB1 and CB2 receptors and the main psychoactive constituent of *Cannabis*, along with 11-hydroxy- $\Delta^8$ -THC-dimethylheptyl (HU-210), a synthetic compound that displays the highest potency at the CB1 receptor (49). Non-classical cannabinoids are synthetic THC

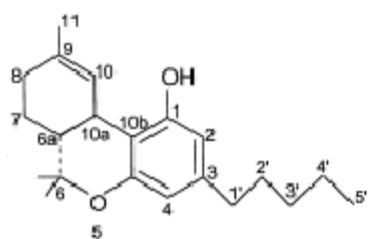
Table 1. The mainly used cannabinoid reagents (35,38)

Main cannabinoid agonists and antagonists	
Ligand	Chemistry
Non-selective cannabinoid receptor agonists	
Anadamide	Eicosanoid derivative, endogenous ligand
2-AG	Eicosanoid derivative, endogenous ligand
HU210	Dibenzopyrane derivative, synthetic
CP55,940	Analog of $\Delta^9$ -THC lacking a pyran ring,
$\Delta^9$ -THC	synthetic
R-(+)-WIN-55,212-2	Dibenzopyrane derivative, plant-derive
	Aminoalkylindole, synthetic
Selective CB1 receptor agonists	
ACEA	Eicosanoid, synthetic
Noladin ether	Lipid-ether, endogenous ligand
R(+)-methanadamide	Eicosanoid, synthetic
ACPA	Eicosanoid, synthetic
O-1812	Eicosanoid, synthetic
Selective CB2 receptor agonists	
JWH-015	Aminoalkylindole, synthetic
JWH-133	Analog of $\Delta^9$ -THC, synthetic
L-759633	Analog of $\Delta^9$ -THC, synthetic
L-759656	Analog of $\Delta^9$ -THC, synthetic
Selective CB1 receptor antagonists	
SR141716A	Diarylpyrazole, synthetic
AM281	Diarylpyrazole, synthetic
SR147778	Diarylpyrazole, synthetic
LY320135	Substituted benzofuranes, synthetic
LH-21	Triazole derivatives, synthetic
AM251	Diarylpyrazole, synthetic
Selective CB2 receptor antagonists	
SR144528	Diarylpyrazole, synthetic
AM630	Aminoalkylindoles, synthetic
Uptake inhibitors	
AM404	Eicosanoid derivatives, synthetic
UCM707	Eicosanoid derivatives, synthetic
AM1172	Eicosanoid derivatives, synthetic
FAAH inhibitors	
OL-135	Alpha heterocycles, syntheric
URB597	O-arylcarbamate, synthetic
PIA	Eicosanoid derivatives, synthetic

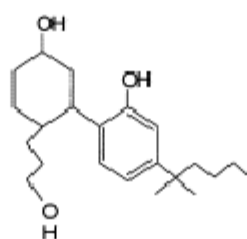
Figure 3. The structure of typical cannabinoid agonists (191).

$\Delta^9$ -THC is a tricyclic dibenzopyran derivatives from plant *Cannabis*. CP-55940 is a synthetic THC analogue that lack the dihydropyran ring. *R*-(+)-WIN-55,212-2 is the represent form of aminoalkylindoles. Endocannabinoids anandamide and 2-AG are eicosanoids.

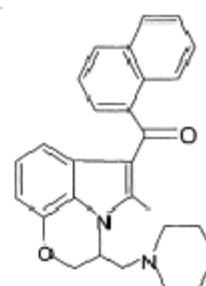




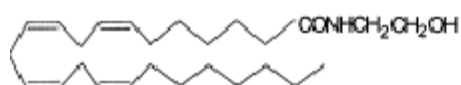
$\Delta^9$ -Tetrahydrocannabinol



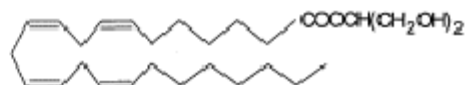
CP-55,940



WIN 55,212-2



Anandamide



2-Arachidonylglycerol

analogues that lack the dihydropyran ring. The most representative form is the Pfizer compound CP-55940, a potent and complete agonist at both the CB1 and CB2 receptors, which was used to characterize the CB1 receptor for the first time (44,51). Aminoalkylindoles were the first non-cannabinoid molecules that displayed cannabimimetic activity (52). *R*-(+)-WIN-55,212-2 is the most representative form, and it behaves as a complete agonist at both the CB1 and CB2 receptors, with higher intrinsic activity at the CB2 receptor. Eicosanoids are the prototypic endocannabinoids, of which anandamide and 2-AG are the most representative compounds. Based on the structure of anandamide, minor chemical changes have led to the development of the first generation of CB1-selective agonists, of which *R*(+)-methanandamide and arachidonyl-2'-chloroethylamide (ACEA) are the most representative forms (50).

#### *Cannabinoid receptor antagonists*

Several compounds have been developed as cannabinoid receptor antagonists such as diarylpyrazoles, substituted benzofuranes, aminoalkylindoles and triazole derivatives (53). Diarylpyrazoles include both the first CB1 receptor antagonist SR 141716A (54) and the first CB2 receptor antagonist SR 144528. A CB2 receptor antagonist, AM 630, belong to aminoalkylindoles (49).

#### *Uptake blockers and inhibitors of fatty acid amide hydrolase*

Based on the structure of anandamide, a series of eicosanoid derivatives that have the ability to block anandamide transport have been synthesized (53). The first and best studied transport inhibitor is AM 404 (55). The administration of AM 404 results in the

accumulation of anandamide and potentiates the effects of exogenously administered anandamide. The compound AM 404 can be degraded by FAAH and behaves as an agonist of vanilloid receptors. UCM 707 (56,57) and AM 1172 (58) have also been shown to efficiently block endocannabinoid uptake. In addition, OL-135 has been shown to possess very high potency and selectivity to reversibly inhibit FAAH activity *in vivo* and *in vitro* (59).

## **Endocannabinoid signaling**

### *CB1 signaling transduction pathways*

The CB1 cannabinoid receptor is a member of the rhodopsin subfamily of GPCRs, which is coupled through Gi/o proteins, negatively to adenylate cyclase and positively to mitogen-activated protein kinase. CB1 receptor stimulation is also coupled to PLC activation, in turn increasing levels of InsP3 for the induction of Ca<sup>2+</sup> release from internal stores (60). CB1 receptors can also interact with Gs to activate adenylate cyclase under conditions of PTX treatment that prevents the receptor interaction with Gi/o proteins (61,62) and in human embryonic kidney (HEK) 293 cells transfected with CB1 and D2 receptors (63).

In addition, CB1 receptors are coupled through Gi/o proteins to certain ion channels, activating G-protein-coupled inward rectifier K<sup>+</sup> (64) and A-type outward potassium channels (65), and inhibiting D-type outward potassium channels (66), N-type and P/Q type calcium channels (67,68), L-type Ca<sup>2+</sup> currents (69). Regardless of the specific target, the actions of cannabinoids are predicted to have an inhibitory effect on neurons in most cases. Inhibition of presynaptic calcium channels reduces neurotransmitter release, whereas activation of postsynaptic K<sup>+</sup> channels suppresses action-potential firing (70).

### *CB2 signaling transduction pathways*

Similar to CB1, CB2 receptors activation can inhibit adenylyl cyclase (71,72) and activate p42/p44 MAP kinase activity, through their ability to couple to Gi/o proteins in CHO cells and HL-60 cells (73,74). Treatment of human prostate epithelial PC-3 cells with cannabinoids activated the PI3K/PKB pathway, which in turn induced translocation of Raf-1 to the membrane and phosphorylation of p42/p44 MAP kinase through CB2 receptor (75). In addition, anandamide could initiate a rise in  $[Ca^{2+}]_i$  in calf pulmonary endothelial cells through activation of PLC, which was sensitive to inhibition by the CB2 antagonist (76), suggesting CB2 receptor was involved in this process.

However, in contrast to CB1, CB2 receptor stimulation was not found to modulate ion channel function in AtT-20 cells transfected with CB2 (77) and *Xenopus* oocytes transfected with CB2 and GIRK1/4 (78).

## **Cannabinoids in gut**

### *Cannabinoid receptors in gastrointestinal tract*

The presence of cannabinoid receptors in the gastrointestinal tract has been demonstrated by anatomical and functional evidences, which was summarized in table 2.

CB1 receptors were detected in enteric nervous system of different species, including mice, rats, guinea pigs, pigs and humans by immunohistochemistry (79-88), where it is frequently colocalized to ChAT neurons. Interestingly, they were not coexpressed in NOS-positive neurons in pig and mice (79,84,85).

CB2 receptor expression was present on plasma cells in the lamina propria and macrophages in human colon (88). Later it was detected in enteric neurons

Table 2. Localization of cannabinoid CB1 and CB2 receptors in the gastrointestinal tract.

Localization of cannabinoid CB1 and CB2 receptors in the gastrointestinal tract.			
Animal species	Region of the gut	Technique	Findings
Pig <sup>(22)</sup>	Ileum	IHC	All CB1 colocalized with ChAT CB1 occasionally associated with SP CB1 co-localised with opioid receptor CB1 not with VIP or NOS-positive neurons
	Colon	IHC	Most CB1 colocalized with ChAT CB1/ChAT-neurons frequently expressed SP in submucosal plexuses CB1 not with VIP or NOS-positive neurons
Guinea pig <sup>(21)</sup>	Myenteric plexus	IHC	Sensory, interneuronal and motoneuronal cell bodies and nerve fibres expressed CB1
Guinea pig <sup>(37)</sup>	Submucosal plexus of ileum	IHC	CB1 colocalized with VIP and NPY CB1 colocalized with TRPV1 on paravascular nerves and fibers in the submucosal plexus
Rat <sup>(33)</sup>	Stomach fundus, corpus and antrum.	IHC	CB1 colocalized with CHAT in neural elements innervating smooth muscle, mucosa and submucosal blood vessels CB2 not observed
Rat <sup>(21)</sup>	Whole mounts of myenteric preparations	IHC	CB1 expressed on cholinergic sensory, interneuronal, and motor neurons
Mice <sup>(24)</sup>	All regions	Immunoblotting	CB1 high in stomach and colon, but not in the pyloric valve
		IHC	CB1 in ganglia subadjacent to the gastric epithelium and in the smooth muscle layers of both the small and large intestine.
		Radioligand binding	[3H]CP 55,940 specific binding in the small intestine
Mice <sup>(31,32)</sup>	Colon	IHC	CB1 in neurons within myenteric and submucosal ganglia and nerve fibers CB1 frequently colocalized to a subpopulation of CHAT neurons and fiber bundles in the myenteric plexus CB1-R and NOS not overlap in myenteric or submucosal neurons and fibers
Human <sup>(38)</sup>	Stomach and colon	RT-PCR	CB1 mRNA detected
Human <sup>(39)</sup>	Colon	IHC	CB1 in all major ganglionated nerve plexuses, predominantly within myenteric ganglia. CB1 in the submucosal plexus, mucosa and in serosa CB1 coexpressed with CHAT in myenteric neurones CB1-R/ChAT nerve fibers in CM layer and submucosa
Human <sup>(40)</sup>	Colon	IHC	CB1 evident in epithelium, smooth muscle, and the submucosal myenteric plexus CB1 on plasma cells in the lamina propria CB2 on plasma cells in the lamina propria and macrophages

morphologically and functionally (89). In addition, CB2R has been demonstrated to reside on a wide variety of peripheral blood leukocytes and spleen cell populations (90) and to a greater extent than seen for CB1R in the same cell types that include B cells, T cells, NK cells, splenic macrophages, dendritic cells, and neutrophils (91-93).

#### *Physiological role of cannabinoids and CB receptors in GI*

Activation of CB1 receptor has been reported to inhibit pentagastrin-induced gastric acid secretion (82,94) at peripheral level. I.c.v. injection of anandamide stimulated gastric acid secretion, however, the response was inhibited by an antagonist of TRPV1 and in the capsaicin-treated rats, but not by an antagonist of cannabinoid receptors (95). It suggested that anandamide could activate TRPV1 in the brain and stimulates gastric acid secretion in rats.

Generally cannabinoids mediate an inhibitory effect on GI motility. Functional studies have shown that cannabinoids inhibited (via CB1 activation) lower oesophageal sphincter relaxation in dogs (96) and ferrets (97) which could be mediated by modulation of vagal activity at peripheral and central levels. Intravenous  $\Delta^9$ -THC administration slows down the rate of gastric emptying of solid food in humans (98). In addition, several cannabinoid agonists inhibited gastric and intestinal motility in rats and mice through CB1 activation (99-101). The antipropulsive effects of cannabinoids could be the result of inhibition of both excitatory cholinergic/tachykininergic and inhibitory VIPergic motor neurons which mediate ascending contraction and descending relaxation respectively, as well as inhibition of the intrinsic sensory CGRP-containing neurons which initiate the peristaltic reflex underlying propulsive activity (102).

*Pathological role of cannabinoids and CB receptors in GI* (Summarized in table 3)

**Inflammation**

Inflammation represents the response of body tissue to immune reactions, injury, or ischemic damage (103). It can be divided into two basic patterns: acute and chronic (104,105). Acute inflammation is of relatively short duration, lasting from a few minutes to several days, and is characterized by the exudation of fluid and plasma components and emigration of leukocytes, predominantly neutrophils, into the extravascular tissues. Chronic inflammation is of a longer duration, lasting for days to years, and is associated with the presence of lymphocytes and macrophages, proliferation of blood vessels, fibrosis, and tissue necrosis (103).

Acute inflammation is the early (almost immediate) reaction of local tissue and their blood vessels to injury. It typically occurs before the immune response becomes established and is aimed primarily at removing the injurious agent and limiting the extent of tissue damage. Acute inflammation involves two major components: the vascular and cellular stages (104-106). The vascular, or hemodynamic, changes are initiated by a momentary constriction of small blood vessels in that area, which is followed rapidly by vasodilatation of the arterioles and venules that supply the area. Accompanying this response is an increased permeability of vessels in the microcirculation, with the outpouring of a protein-rich fluid (exudate) into the extravascular spaces. The cellular stage of acute inflammation is marked by movement of leukocytes into the area of injury. Two types of leukocytes participate in the acute inflammatory response-granulocytes and monocytes (103).

A rapid response also requires the release of chemical mediators from tissue cells (mast



Table 3. The role of cannabinoids and cannabinoid receptors in inflammation of gastrointestinal tract.

The role of cannabinoids and cannabinoid receptors in inflammation of gastrointestinal tract	
Experimental models /animal species	Functions
<b>Ileum</b>	
Rat <sup>(63)</sup>	Anandamide and 2-AG caused ileitis via VR1 and SP release WIN 55,212-2, HU 210 and PEA had no affect
Indomethacin-induced small intestinal injury (rat) <sup>(66)</sup>	Oral CB1 antagonist prevented ulcers and the rise in TNF- $\alpha$ levels and intestinal MPO activity. Oral CB2 antagonist prevented intestinal ulcers only (+)-WIN 55, 212-2 and CP 55940 had no effects
LPS-induced ileitis (rat) <sup>(66)</sup>	Oral CB1 antagonist prevented the increase of TNF-a levels CB2 antagonist had no effect
LPS-induced ileitis (WT and CB1-/- mice) <sup>(66)</sup>	Oral CB1 antagonist inhibited the increase of TNF-a in WT, but not in CB1-/- mice CB2 antagonist had no effect in both WT and CB1-/- mice
Croton oil-induced intestinal inflammation (mice) <sup>(68)</sup>	Cannabinoids (i.p.) were more active in delaying intestinal motility via peripheral CB1 receptors
LPS-induced ileitis (rat) <sup>(70)</sup>	CB2 agonist inhibits intestinal motility via cyclooxygenase, IL-1 $\beta$ and eNOS.
<b>Colon</b>	
Intracolonic OM induced acute colitis (mice) <sup>(75)</sup>	CB1 and CB2 agonists (i.p.) attenuate OM colitis CB1R upregulated in endothelium and myenteric ganglia CB2R-positive cells were evident
DSS induced colitis (mice) <sup>(75)</sup>	CB1 and CB2 agonists attenuated DSS colitis CB1R upregulated in myenteric ganglia CB2R immunoreactivity evident
DNBS and DSS colitis (WT and CB1-/- mice) <sup>(77)</sup>	DNBS and DSS induced stronger inflammation in CB1-/- mice Cannabinoid agonist HU210 or FAAH-/- protect against DNBS colitis Myenteric neurons expressing CB1 increased in DNBS colitis
DNBS colitis (CB1-/-, TRPV1-/- and WT mice) <sup>(78)</sup>	Rhythmic action potentials in colonic circular smooth muscle cells of CB1-/- and TRPV1-/- mice IJP in CB1-/- mice was longer
DNBS colitis (mice) TNBS colitis (rat) Ulcerative colitis (human) <sup>(81)</sup>	Elevation of anandamide Inhibitor of anandamide reuptake (VDM11) abolished inflammation Inhibitor of FAAH (AA-5-HT) less efficacious at attenuating colitis
TNBS colitis (rat) <sup>(82)</sup>	CB1 and CB2 agonists (i.p.) reduce basal sensitivity and TNBS colitis-induced hypersensitivity to CRD CB1 antagonist enhanced colitis-induced hyperalgesia.
Rats and mice <sup>(83)</sup>	probiotic bacteria NCFM induced the expression of MOR1 (m-opioid receptors) and CB2 in epithelial cells of colonic section
Butyrate enemas elicited colonic hypersensitivity (rat) <sup>(83)</sup>	NCFM-induced analgesia was inhibited by CB2-selective antagonist
Human colonic epithelial cell lines <sup>(40)</sup>	Cannabinoids enhanced epithelial wound closure by the CB1 receptor
Inflammatory bowel disease (human) <sup>(40)</sup>	CB2 immunoreactivity in the epithelium of colonic tissue

cells and macrophages) that are prepositioned in the tissue (107). Mast cells are particularly prevalent along mucosal surface of the lung and gastrointestinal tract and the dermis of the skin. This distribution places the mast cell in a sentinel position between environmental antigens and the host for a variety of acute and chronic inflammation conditions (105).

Although inflammation is precipitated by injury, its signs and symptoms are produced by chemical mediators that are derived either from the plasma or from cells. Plasma-derived mediators of inflammation include the kinins, the products of the coagulation/fibrinolysis system, and the proteins of the complement system. Cell-derived mediators include histamine and serotonin, arachidonic acid metabolites, platelet-activating factors, cytokines, and nitric oxide (103).

Characteristic of chronic inflammation is an infiltration by mononuclear cells (macrophages) and lymphocytes instead of the neutrophils commonly seen in acute inflammation. Chronic inflammation also involves the proliferation of fibroblasts instead of exudates (103).

#### *Role in ileum inflammation (Figure 4)*

Cannabinoids in gut inflammation have been widely studied in experimental animal models. Endocannabinoids cause inflammation in the rat ileum via capsaicin VR1 receptor-mediated SP release from primary sensory nerves in the intestinal mucosa. Toxin A increased tissue concentrations of anandamide and 2-AG in the ileum, and these effects were enhanced after pretreatment with inhibitors of fatty acid amide hydrolase, suggesting endocannabinoids may mediate the inflammatory effects of toxin A (108).

Figure 4. Role of cannabinoids and cannabinoid receptors in ileitis.

Continuous arrows denote stimulation and induction. Broken arrows denote inhibition.

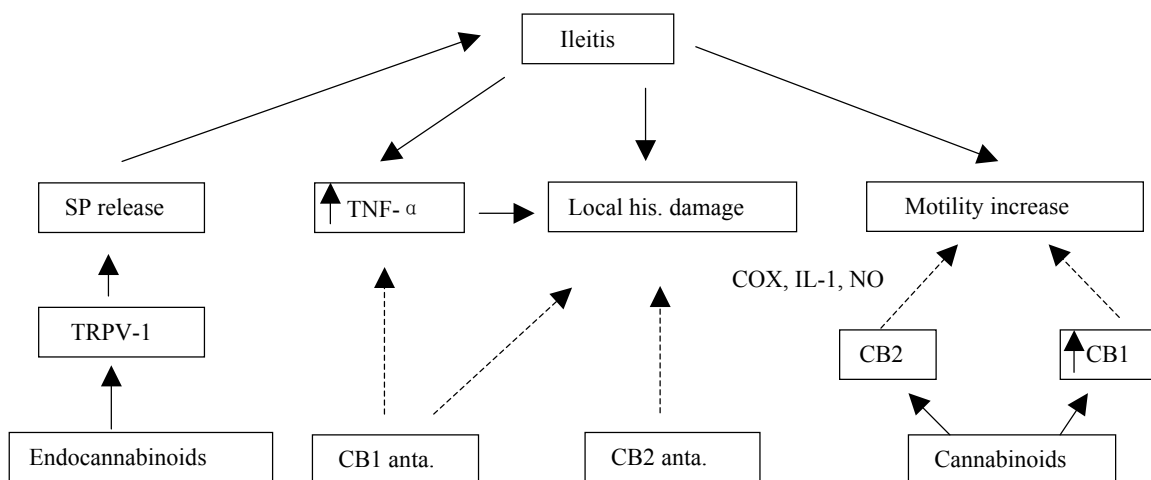


Figure 4. Roles of cannabinoids and cannabinoid receptors in ileitis.  
Continuous arrows denote stimulation and induction. Broken arrows denote inhibition.

Substantial studies have shown that cannabinoid antagonists play a protective role in inhibition of mucosa damage via both CB1 and CB2 receptors. CB1 antagonists dose-dependently prevented indomethacin-induced ulcers, the rise in TNF- $\alpha$  levels and intestinal MPO activity. CB2 antagonists prevented intestinal ulcers only. In addition, CB1 antagonists prevented LPS-induced increase of TNF- $\alpha$  levels in rat and wide-type mice plasma, but not in CB1 receptor knockout mice (109), suggesting that the anti-inflammatory role of CB1 antagonists was mainly mediated by cytokine TNF- $\alpha$ .

Cannabinoids (i.p.) were more active in delaying intestinal motility in croton oil-treated mice than in control mice which was reversed by CB1 antagonist, indicating an involvement of CB1. Croton oil-induced intestinal inflammation was associated with an increased expression of CB1 receptor. This up-regulation explains the increased potency of exogenous cannabinoid agonists during inflammation (110). CB2 receptor agonist reduced lipopolysaccharide (LPS) stimulated gastrointestinal transit back to control values, and this inhibition was completely prevented by the CB2 receptor antagonist, which itself was without effect. The effects of the CB2 agonist were found to act via cyclooxygenase in that inhibition of cyclooxygenase with indomethacin completely abrogated the inhibitory effect of CB2 agonist. In addition, IL-1  $\beta$  and constitutive NOS isoforms (probably eNOS) may be involved in this effect (111).

In summary, cannabinoids may participate in mucosa damage but delay motility in intestinal inflammation via both CB1 and CB2 receptors.

#### Role in colitis (Figure 5)

The evidence supported that cannabinoids mediate a protective role in experimental colitis via both CB1 and CB2 receptors. For example, studies have shown that

intraperitoneal application of the CB1R-selective agonist ACEA and the CB2R-selective agonist JWH-133 inhibited Oil of mustard (OM)- and dextran sulfate sodium (DSS)-induced colitis (112). Inhibitors of anandamide reuptake (VDM11) significantly elevated anandamide levels in the colon of DNBS-treated mice and concomitantly abolished inflammation (113). In addition, both intrarectal infusion of 2,4-dinitrobenzene sulfonic acid (DNBS) and oral administration of dextrane sulfate sodium (DSS) induced stronger inflammation in CB1-deficient mice (*CB1*<sup>-/-</sup>) than in wild-type littermates (*CB1*<sup>+/+</sup>), and treatment of wild-type mice with the CB1 antagonist mimicked the phenotype of *CB1*<sup>-/-</sup> mice, indicating a protective role of the CB1 receptors during inflammation. Consistently, treatment with the cannabinoid receptor agonist HU210 or genetic ablation of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) resulted in protection against DNBS-induced colitis (114). In addition, cannabinoids enhanced epithelial wound closure in human colonic epithelial cell lines by the CB1 receptor (88). During colitis, the endocannabinoid system was also upregulated. Studies showed that the upregulation of CB1 in endothelium and myenteric ganglia, and also CB2R-positive cells were evident as a dense, intensely stained infiltrate in OM and DSS colitis tissue (85). DNBS treatment also increased the percentage of myenteric neurons expressing CB1 receptors (114). CB2 immunoreactivity was seen in the epithelium of colonic tissue characteristic of inflammatory bowel disease (88). A strong elevation of anandamide, but not 2-AG, levels was found in the colon of DNBS-treated mice, in the colon submucosa of TNBS-treated rats, and in the biopsies of patients with ulcerative colitis, suggesting the protective role of anandamide in colitis and IBDs (113).

In addition, activation of CB1 and CB2 receptors could play an analgesic role in colon

Figure 5. Roles of Cannabinoids and cannabinoid receptors in colitis.

Continuous arrows denote stimulation and induction. Broken arrows denote inhibition.



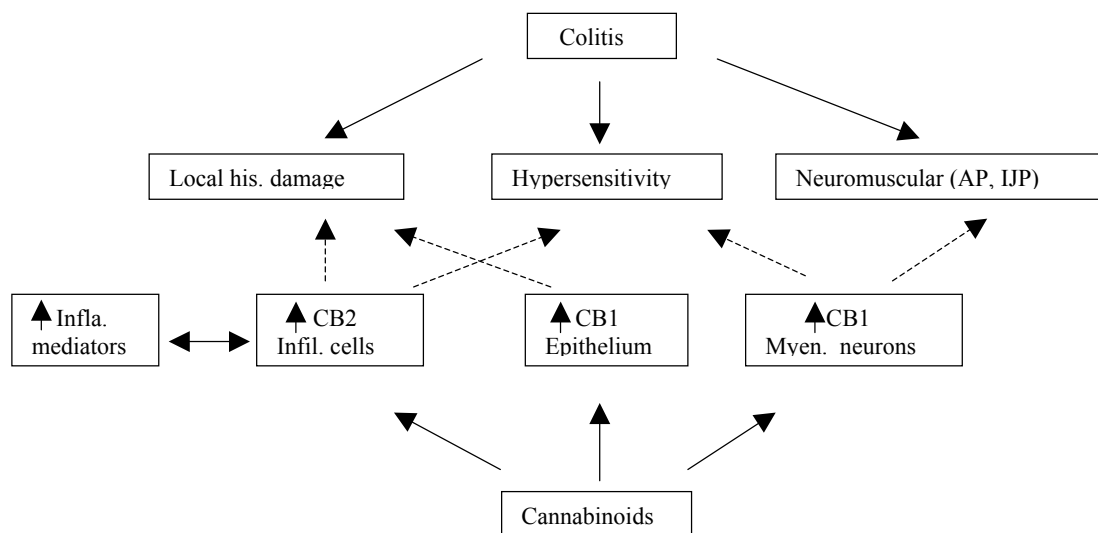


Figure 5. Roles of Cannabinoids and cannabinoid receptors in colitis. Continuous arrows denote stimulation and induction. Broken arrows denote inhibition.

inflammation. Studies found that activation of either CB1 or CB2 receptors reduces the basal sensitivity and the TNBS colitis-induced hypersensitivity to colorectal distension (CRD) in rats, both agonists being more active in the presence of colitis. These effects were blocked by their antagonists respectively. CB1 receptor antagonist enhanced colitis-induced hyperalgesia, suggesting the endogenous cannabinoid system is involved in the inflammatory hyperalgesia through CB1 receptors (115). In a model of chronic colonic hypersensitivity, elicited by butyrate enemas and mimicking irritable bowel syndrome, treatment with probiotic bacteria NCFM significantly increased the colorectal distension threshold which was inhibited by CB2-selective antagonist; in addition, administration of NCFM significantly induced the expression of CB2 in epithelial cells of colonic section of rats and mice, providing indirect evidence for a physiological role of CB2 in the control of intestinal pain (116).

In summary, cannabinoids play a protective role in inflammatory damage and analgesic role in colitis via both CB1 and CB2 receptors.

### **Cannabinoids in central nervous system**

The role of cannabinoids in the brain has been well studied. Cannabinoids suppressed neuronal activity via CB1 presynaptic inhibition of neurotransmitters release in hippocampal and cerebellar neurons (117-123). In addition, endocannabinoids were involved in regulation of food intake (124), inhibition of emesis (125) and analgesia (126,127).

The effect of cannabinoids on neuronal survival has been studied by many groups, however, the results are controversial varying from neuroprotection (128-132) to neurotoxicity (133-135). For example, cannabinoids mediate a neuroprotective effects in

neurotoxin S-AMPA induced primary cortical neurons death (131). Moreover, the endocannabinoid system is highly activated during CNS inflammation and anandamide protects neurons from inflammatory damage via CB1/2 receptors (130). In contrast, anandamide was also found to induce cell death in primary neuronal cultures via calpain and caspase pathways (134).  $\Delta^9$ -THC induced apoptosis in cultured rat cortical neurons via P53 (135).

### **Hypothesis**

Although the major effects of cannabinoids in the gut are mediated through effects on enteric neurons, the role of cannabinoids in enteric nervous system is poorly understood. Given the importance of cannabinoids in inflammation of gut and their effects in neuronal survival and death in CNS, we hypothesize that cannabinoids could affect enteric neuronal survival and we will identify the pathways involved. Since the endocannabinoid system is upregulated in inflammatory bowel diseases, this effect may play a role in the pathogenesis of the response to inflammation as well as the recovery and reinnervation of the gut following the acute phase of inflammation. The further significance of this work could contribute to developing new therapeutic methods for inflammatory bowel disease and related symptoms in clinic practice.

## **Materials and Methods**

### **Materials**

#### *Materials for cell culture*

Hartley guinea pigs (150-200g, male) were purchased from Charles River Laboratories (Wilmington, MA). All procedures with animals were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Dulbecco Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Frederick, MD). 10% fetal bovine serum (FBS) was purchased from Atlas (Tartu, Estonia). The fetal enteric neuronal cell line was a kind gift from Dr. Srinivasan's group (University of Emory). DMEM-F12, N2 medium, Neurobasal A medium, B-27 serum-free supplement, 0.25% Trypsin-EDTA were purchased from Invitrogen (Frederick, MA). rhGDNF was purchased from Promega (Madison, WI). C8-D1A (Astrocyte type I clone) was purchased from ATCC (Manassas, VA).

#### *Antibodies*

Monoclonal to PGP9.5 (Mouse) was purchased from Abcam Inc. (Cambridge, MA). CB1 (N-15) (Goat) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-protein gene product 9.5 (PGP9.5) (Rabbit), anti-S-100 protein monoclonal antibody (Mouse) and anti-cannabinoid receptor 2, N-terminus (Rabbit) were purchased from Millipore (Billerica, MA). Alexa Fluor 488 (donkey anti-mouse) and Alexa Fluor 594 (donkey anti-goat, donkey anti-guinea pig and donkey anti-rabbit) are purchased from Invitrogen (Frederick, MA).

Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (rabbit) and p44/42 MAPK

(Erk1/2) antibody (rabbit) were purchased from Cell signaling (Danvers, MA). Monoclonal anti- $\beta$ -actin (mouse, 1:10000) was purchased from Sigma (St. Louis, MO). Anti-CB1 antibody (rabbit) was purchased from Affinity Bioreagents (Golden, CO). HRP conjugated 2<sup>nd</sup> antibodies: goat anti-rabbit (1:1000) and goat anti-mouse (1:1000) and Supersignal west femto chemiluminescent substrate were purchased from Pierce (Rockford, IL). Odyssey 2<sup>nd</sup> antibodies: rabbit (1:10000) and mouse (1:10000) were purchased from Licor Biosciences (Lincoln, NE).

#### *Other chemicals*

Vectastain elite ABC kit (rabbit IgG) and Peroxidase substrate 3, 3' Diaminobenzidine (DAB) kit were purchased from Vector Laboratories, Inc. (Burlingame, CA). Protease inhibitor cocktail was purchased from BD Biosciences (Palo Alto, CA). Protein assay kit was purchased from Biorad (Hercules, CA). AM251, AM630, Capsazepine, MAP kinase kinase (MEK) inhibitor PD98059, Phospholipase C (PLC) inhibitor U73122, Phosphatidylinositol-3-kinase (PI-3K) inhibitor LY 294002 and other chemicals were purchased from Sigma (St. Louis, MO).

## **Methods**

#### *Collection of tissue*

Guinea pigs were sacrificed by CO<sub>2</sub> asphyxiation. The small intestine was dissected out immediately and placed in 37°C oxygenated Krebs solution of the following composition: NaCl 118 mM, KCl 4.75 mM, KH<sub>2</sub>PO<sub>4</sub> 1.19 mM, MgSO<sub>4</sub> 1.2 mM, CaCl<sub>2</sub> 2.54 mM, NaHCO<sub>3</sub> 25 mM and Dextrose 11 mM (pH 7.4).

### *Preparations of ganglia from guinea pig myenteric plexus*

The tissue was cut into 3-4 cm sections for preparation of myenteric ganglia by the method of Yau (138). The segments of intestine were placed on glass rods and the sheets of longitudinal muscle with the myenteric plexus were scrapped with a wet Kim wipe and incubated for 6 minutes at 37°C in Krebs-bicarbonate solution containing 0.1% protease, 0.2% collagenase type IV and 0.1% BSA for enzymatic digestion. The tissue was consistently bubbled with 95%O<sub>2</sub>/5%CO<sub>2</sub>, accompanied by frequently suctioning with 5 ml pipette to free the ganglia from the smooth muscle cells during this process. The partly digested tissue was then collected by filtering through 500-um Nitex mesh and incubated for one hour at 37°C in enzyme-free Krebs solution bubbling with 95%O<sub>2</sub>/5%CO<sub>2</sub> for further isolation. The suspension was then filtered to remove the undigested tissue and the filtrate was centrifuged for 1 minute at 1000 RPM. The supernatant containing smooth muscle cells and debris was discarded, and the pellet containing the ganglia was resuspended and washed twice in enzyme-free Krebs solution to remove cell debris. Then the pellet was resuspended and placed in a 10cm plastic culture dish and the ganglia were harvested by suction into 20ul capillary tube under dissecting microscope.

### *Cultures in plates for treatment with anandamide and other reagents*

Ganglia were placed in 24 well plates with a density of 15 ganglia per well, and incubated for three days in Dulbecco Modified Eagle's Medium (DMEM) containing 200U/ml penicillin, 200ug/ml streptomycin, 100ug/ml gentamycin, 2.5 ug/ml amphotericin B and 10% fetal bovine serum (DMEM10) at 37°C 10%CO<sub>2</sub> incubator. In some experiments, the ganglia were incubated in DMEM10 containing 10uM of mitotic inhibitor arabinoside cytosine (araC) to reduce the glial and other nonneural cell effects on the neurons. After

three days incubation, the ganglia attached to the bottom of the plates. The wells were washed two times with serum free medium (DMEM0) containing 200U/ml penicillin, 200ug/ml streptomycin, 2.5ug/ml amphotericin B, and 100ug/ml gentamycin and treated with anandamide (1nM-10uM) and/or the CB1 antagonist AM251 (1uM) or CB2 antagonist AM630 (1uM) or TRPV1 antagonist capsazepine (1uM) in serum free medium DMEM0 or DMEM0 with 10uM araC for four days. In one set of experiments, the ganglia were treated with 0.1uM anandamide and/or MAP kinase kinase (MEK) inhibitor PD98059 (1uM) or phospholipase C (PLC) inhibitor U73122 (1uM) or phosphatidylinositol-3-kinase (PI-3K) inhibitor LY 294002 (1uM). Untreated wells were taken as a control. Duplicates were done for each treatment in each animal. The plates were then prepared for immunohistochemistry.

### *Immunohistochemistry*

The plates were washed three times with phosphate buffer saline (PBS) solution containing 137mM NaCl, 2.682 mM KCl, 3.895 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.764mM KH<sub>2</sub>PO<sub>4</sub> at 4 °C, and fixed with 4% paraformaldehyde with the following composition: 0.04g/ml paraformaldehyde, 2.5mM NaOH, 28mM NaH<sub>2</sub>PO<sub>4</sub> and 37.4mM Na<sub>2</sub>HPO<sub>4</sub> for 30 minutes. The cells were then washed twice with PBS, and incubated with 0.1% triton X-100 for 10 minutes. After blocking with normal goat serum (NGS) from VECTASTAIN ABC kit (Vector Laboratories) for 20 minutes, the plates were incubated with primary antibody to protein gene product 9.5 (anti-PGP9.5) (rabbit, Millipore Corp.) diluted 1:1000 in PBS containing 1% blocking serum at 4 °C for 24 hours.

On the second day, the cultures were washed three times with PBS and incubated with the

biotinylated secondary antibody (goat anti-rabbit) for 30 minutes. After washing with PBS for 5 minutes, wells were incubated with the conjugate of Avidin/Biotinylated horseradish peroxidase (HRP) complex (VECTASTAIN ABC kit, Vector Laboratories) for 30 minutes. Then washing once with PBS for 5 minutes, the ganglia were incubated with 3, 3'-Diaminodenzidine (DAB) working solution from DAB kit (Vector Laboratories, Inc.) for 4-10 minutes. After washing with distilled water for 5 minutes, the wells were covered with Crystal/Mount (Biomedica Corp.) and placed at 70 °C for 10 minutes to dry. These plates were examined under an inverted light microscope.

#### *Counting of neurons and ganglia and calculations*

After staining with the neuronal marker anti-PGP9.5 antibody, the number of ganglia in each well and the number of neurons in each ganglion were counted. The total number of neurons was calculated by adding the number of neurons in each ganglion together in each well. The percentage changes were calculated through the control divided by the treated groups. The survivals were expressed as the mean of percentage change from control  $\pm$  standard error of the mean (SEM).

#### *Cultures for immunofluorescence staining*

In this protocol, ganglia were placed in 8 well slides with a density of 15 ganglia per well, and incubated in DMEM10 medium for seven days at 37°C 10%CO<sub>2</sub> incubator. The medium was replaced on day 3. Then the ganglia were prepared for immunofluorescence staining.



### *Immunofluorescence staining*

After culture of ganglia on 8 well slides for seven days, the slides were washed three times with IHC solution containing 26mM  $\text{NaH}_2\text{PO}_4$  and 39mM  $\text{Na}_2\text{HPO}_4$  and fixed with 4% paraformaldehyde as described above for 30 minutes. After washing two times with IHC solution, the ganglia were treated with 0.3% Triton X-100 for 10 minutes to permeabilized the cells and incubated for 20 minutes with 3% donkey normal serum (DNS) to block the nonspecific binding of the antibody. The cultures were then incubated with the primary antibodies to PGP9.5 (mouse, Abcam) (1:500), S-100 (mouse, Chemicon) (1:100), CB1 (goat, Santa cruz) (1:100), and CB2 (rabbit, Millipore) (1:100) at 4 °C for 24 hours. All primary antibodies were diluted with 3% DNS to inhibit the nonspecific binding. Negative controls were performed by deleting the primary antibodies.

On the second day, the slides were washed three times with IHC and incubated two hours in dark place at room temperature with the secondary antibodies conjugated to either Alexa Fluor 488 (1:500) or Alexa Fluo 594 (1:500). The secondary antibodies were determined according to the species of the primary. After incubation, the slides were washed three times with IHC solution, covered with permaflour, covered with a glass coverslip, and placed in the dark place for 24 hours to dry. Then slides were sealed with nail polish and examined under the microscope with appropriate filters for Alexa Fluor 488 or Alexa Fluor 594.

### *Cultures for enteric neuronal cell line*

The enteric neuronal cell line was cultured by the method of Anitha et al (139). The

frozen cells were thawed at 37°C for 2-3 minutes, suspended in 10ml N2 medium with the following composition: DMEM-F12 medium, N2 medium, 10ng/ml GDNF, 10% FBS, 200U/ml penicillin and 200ug/ml streptomycin and centrifuged for 5 min at 130 x g to remove DMSO. The cells were then resuspended in N2 medium and placed onto T-25 flasks. The flasks were cultured in a humidified incubator containing 10%CO<sub>2</sub> at permissive temperature 33 °C for proliferation until confluence was attained. The cells in flask were then dislodged mechanically by trypsin-ethylenediaminetetraacetic acid (0.25%) and re-plated on 8 well slides and/or 6 well plates at a concentration of  $5 \times 10^5$  cells/ml in N2 medium. The cells were then incubated for the following protocols. All experiments were performed between passages 13 and 40 and duplicates were done for each treatment in each experiment.

*Non-treated wells for immunofluorescence staining*

The 8 well slides were cultured at 33 °C for 2-3 days, and then move to 5% CO<sub>2</sub> 39 °C incubator in neurobasal-A medium containing B-27 serum-free supplement, 1mmol/L glutamine, 5uM Na<sub>3</sub>VO<sub>4</sub>, 200U/ml penicillin and 200ug/ml streptomycin for 7 days. The medium was replaced once on day 3. Then the slides were prepared for immunofluorescence staining with anti-PGP9.5 (rabbit, 1:1000), anti-CB1 (goat, 1:50) or anti-CB2 (rabbit, 1:200) antibodies as described above.

*Treatment with anandamide at 39 degree for 7 days.*

The 8 well slides and 6 well plates were cultured at 33 °C for 2-3 days, and then treated with anandamide (0, 1nM, 0.1uM, 10uM) diluted in neurobasal-A medium for 7 days at 5% CO<sub>2</sub> 39 °C incubator. The medium was replaced once on day 3. The slides were then immunostained with anti-PGP9.5 antibody (rabbit, Millipore) as described above. The

cells grown in plates were prepared for western blot.

*Treatment with anandamide at 33 degree for 2 days.*

To eliminate the effect of anandamide on cell differentiation at 39 degree, the slides were cultured at 33 °C in N2 medium for 24 hours. After this incubation the cells were settled to the bottom of the slides. The slides were then treated with anandamide (0, 1nM, 0.1uM, 10uM) diluted in N2 medium and continued to incubate at 33 °C for 2 days. The medium of slides was changed to neurobasal-A medium without anandamide and cultured for 7 days at 5% CO<sub>2</sub> 39 °C incubator. The slides were then immunostained with anti-PGP9.5 antibody (rabbit, Millipore) as described above and examined under microscope.

*Coculture of astrocyte cell line with enteric neuronal cell line and treatment with anandamide.*

The astrocyte cell line was thawed in 37°C for 2-3 minutes, transferred to 75cm<sup>2</sup> tissue culture flasks and diluted in DMEM containing 200U/ml penicillin, 200ug/ml streptomycin, 100ug/ml gentamycin, 2.5ug/ml amphotericin B and 10% fetal bovine serum (DMEM10). The flasks were then placed at 5% CO<sub>2</sub> 37 °C incubator until confluence. The cells were dispersed by 0.25% (w/v) trypsin-0.53Mm EDTA solution and re-plated on 8 well slides at a concentration of  $1 \times 10^5$  cells/ml in DMEM10 medium for 24 hours at 5% CO<sub>2</sub> 37 °C incubator. After this incubation, the cells adhered to the bottom of the slides.

Then the medium was removed and the enteric neuronal cell line was plated on these slides at a concentration of  $5 \times 10^5$  cells/ml in N2 medium for 2-3 days at 33°C incubator. The slides cocultured astrocyte cell line and enteric neuronal cell line were then treated

with anandamide (0, 1nM, 0.1uM, 10uM) diluted in neurobasal-A medium for 7 days at 5% CO<sub>2</sub> 39 °C incubator. The medium was replaced once on day 3. The slides were immunostained with anti-PGP9.5 antibody on day 7 (rabbit, Millipore) as described above. Duplicate wells were done for each treatment in each experiment.

#### *Counting of neuronal cells and calculations*

The PGP9.5 immunoreactive cells were identified as the neuronal cells. The number of neuronal cells and the number of total cells were counted in a 0.1215mm<sup>2</sup> grid and three randomly selected fields per well were examined. The neuronal cell percent was calculated using the number of neuronal cells divided by the number of total cells per grid. The percentage changes were calculated through the control divided by the treated groups. Neuronal survival is expressed as the mean of percentage change of PGP9.5-positive cells in total cells  $\pm$ SEM.

In coculture slides, the astrocytes and enteric neuronal cells are easily differentiated and neuronal survival is expressed as the mean of percentage change of PGP9.5-positive cells in total enteric neuronal cells  $\pm$ SEM.

#### *Protein concentration assay and western blot analysis*

##### *Protein extraction*

After treatment with anandamide, the enteric neuronal cells grown in 6 well plates were prepared for protein exaction. The plates were rinsed with cold PBS twice and then incubated in 0.3ml/well lysis buffer solution containing 50mM Tris/Cl, pH 7.5, 150mM NaCl, 0.1%SDS, 0.5% Sodium deoxycholate, 1% NP-40, 10mM Sodium pyrophosphate

and protease inhibitor cocktail (BD Biosciences) containing 16 ug/ml benzamidine HCl, 10 ug/ml phenanthroline, 10ug/ml aprotinin, 10ug/ml leupeptin, 10ug/ml pepstatin A, and 1mM PMSF for 1-2 minutes. The cells were scrapped off and sonicated for 10 seconds to disrupt the cells. The samples were centrifuged for 20 minutes at 4 °C. The supernatant was then transferred to new vials for Biorad protein assay and western blot and the pellet was discarded.

#### Biorad protein assay

The BSA standards (2.0, 1.2, 1.0 0.8, 0.6, 0.4, 0.2 and 0 mg/ml) were used for generating a standard curve. The protein concentration of samples was determined from the X-axis of the standard curve.

#### Western blot

The protein extracts were resolved using 10% SDS/PAGE gel and then transferred onto a nitrocellulose membrane for two hours at 4°C in 100-125 volts. After blocking with 5% milk for one hour, the membranes were incubated with primary antibodies for 24 hours at 4°C. The membranes were then washed three times with Tris Buffered Saline Tween-20 (TBST) buffer and incubated with Odyssey fluorescent secondary antibodies for one hour. After three times washes with TBST, the membranes were developed by Odyssey. The band intensities were quantitated by Odyssey software. For detecting the CB2 expression in enteric neuronal cell line, the horseradish peroxidase-conjugated secondary antibody (1:1000) was used and the protein bands were identified by Supersignal west femto chemiluminescent kit (Thermo scientific).

**Statistics**

The results are expressed as means  $\pm$  SEM. of n experiments and analyzed for statistical significance using Student's *t*-test or ANOVA. The probability of  $P < 0.05$  was considered statistically significant.

## **Results**

### **Ganglion isolation and growth**

The myenteric ganglia were successfully isolated from the guinea pig small intestine, and visualized in phase contrast microscopy (Figure 6, left panel). Then each well of the culture plates was seeded with 15 ganglia and grown in DMEM 10 medium for 3 days; at this stage the ganglia were attached to the floor of culture slides, and glia and neurons began expanding their processes and neurites. Then the ganglia were grown in DMEM0 medium for 4 days and immunostained with an antibody to the general neuronal marker PGP9.5. The enteric neurons can be easily identified after this process as shown in Figure 6 (right panel).

### **Effect of anandamide on ganglionic survival**

After 7 days in culture, the average number of ganglia per well was  $6.05 \pm 0.67$  in the control group (Figure not shown). The results with anandamide treatment indicated that anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentrations (1nM-0.1uM) and decreasing survival at high concentration (10uM). Maximal survival (68% increase in number of ganglia surviving) occurred at 0.1uM and the ED50 was 3nM (Figure 7).

To differentiate which cannabinoid receptor(s) mediated these effects, primary cultures were treated with anandamide and CB1 cannabinoid receptor antagonist, AM251 (1uM).

Figure 6. Images of freshly isolated ganglia (left) and ganglia grown 7 days in culture (right).

Left panel illustrates freshly isolated ganglia from guinea pig intestine and right panel shows a ganglia grown 3 days in DMEM10 and another 4 days in DMEM0 culture medium and immunostained with neuronal marker anti-PGP9.5 antibody.



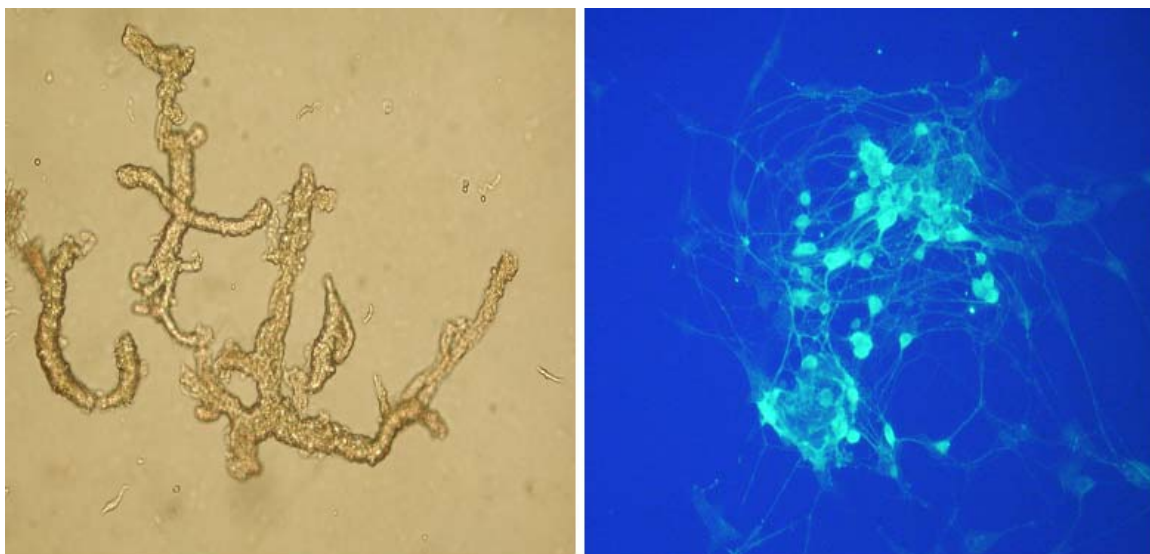
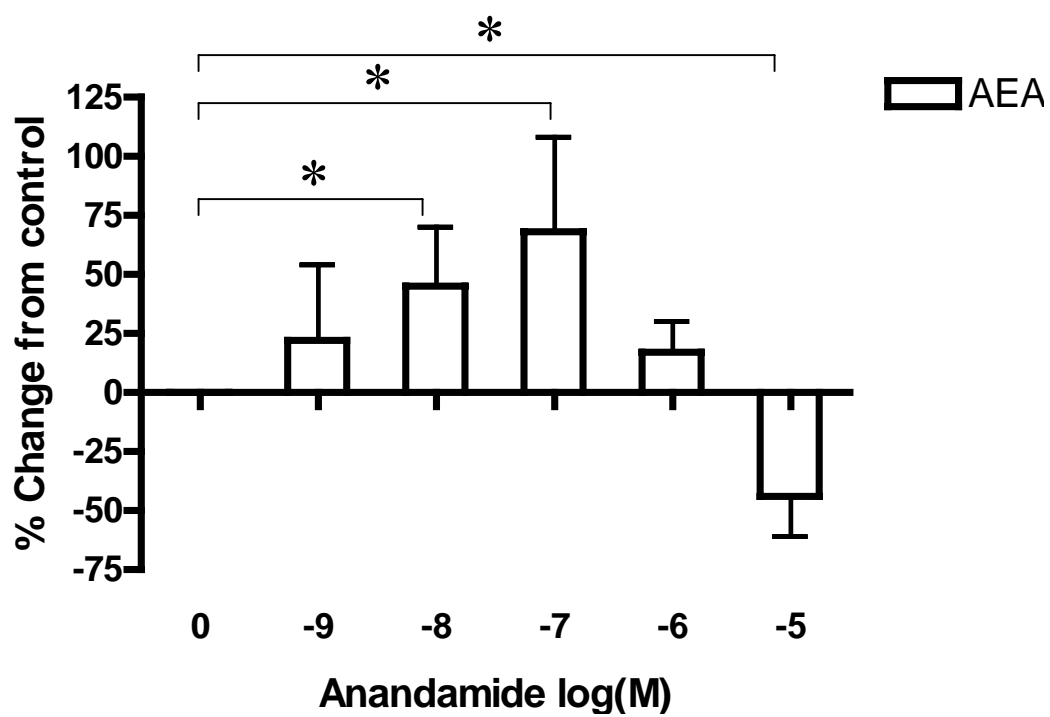


Figure 7. Anandamide dose response curve for ganglia survival in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentrations (1nM-0.1uM) and decreasing survival at high concentration (10uM). Maximal survival (68% increase in number of ganglia surviving) occurred at 0.1uM and the ED50 was 3nM. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals. \* =  $P < 0.05$ .



As shown in Figure 8, anandamide induced increase in survival was inhibited by AM251, suggesting this effect was mediated by CB1 receptors. The CB1 antagonist alone had no effect on the number of ganglia surviving compared with control, suggesting endogenous cannabinoids may not play a role in ganglia survival through CB1 receptor. In addition, AM251 did not block the decreased survival of ganglia induced by the higher concentration of anandamide (10 $\mu$ M). This suggested that the decrease in survival of ganglia was not due to CB1 receptors.

Next we examined whether CB2 receptors play a role in the ganglia survival. We used the CB2 receptor specific antagonist AM630 to address this question. Again, anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentration (0.1 $\mu$ M) and decreasing survival at high concentration (10 $\mu$ M). The effect of anandamide on promoting survival was not inhibited by CB2 antagonist AM630 (1 $\mu$ M); however, the effect on decreasing survival was significantly inhibited by AM630 (Figure 9). These results suggested that high concentration of anandamide activate CB2 receptors and decrease ganglia survival. The results also suggest that CB2 receptor was not involved in the increase in ganglia survival induced by low concentrations of anandamide (0.1 $\mu$ M). The CB2 antagonist alone had no effect on the cultured ganglia survival, suggesting endogenous cannabinoid may not play a role in ganglia survival through CB2 receptor (Figure 9).

The TRPV1 receptor can be found in extrinsic primary sensory neurons as well as intrinsic sensory neurons. There are some evidence that anandamide could activate the TRPV1 receptor, although at high concentrations. Here we tested whether the effect of anandamide on ganglionic survival involved TRPV1. We found that both the

Figure 8. Anandamide dose response curve for ganglia survival with CB1 antagonist in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentration (0.1uM) and decreasing survival at high concentration (10uM). The effect of anandamide on increasing ganglia survival was inhibited by AM251. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals. \* =  $P < 0.05$ .

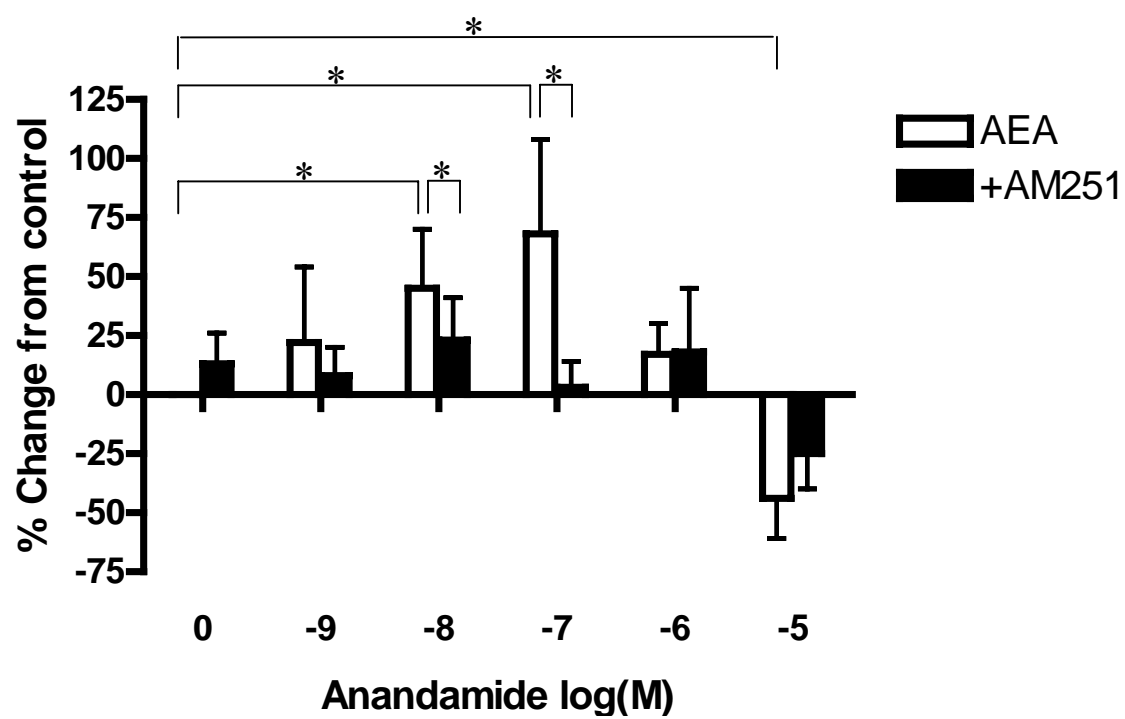
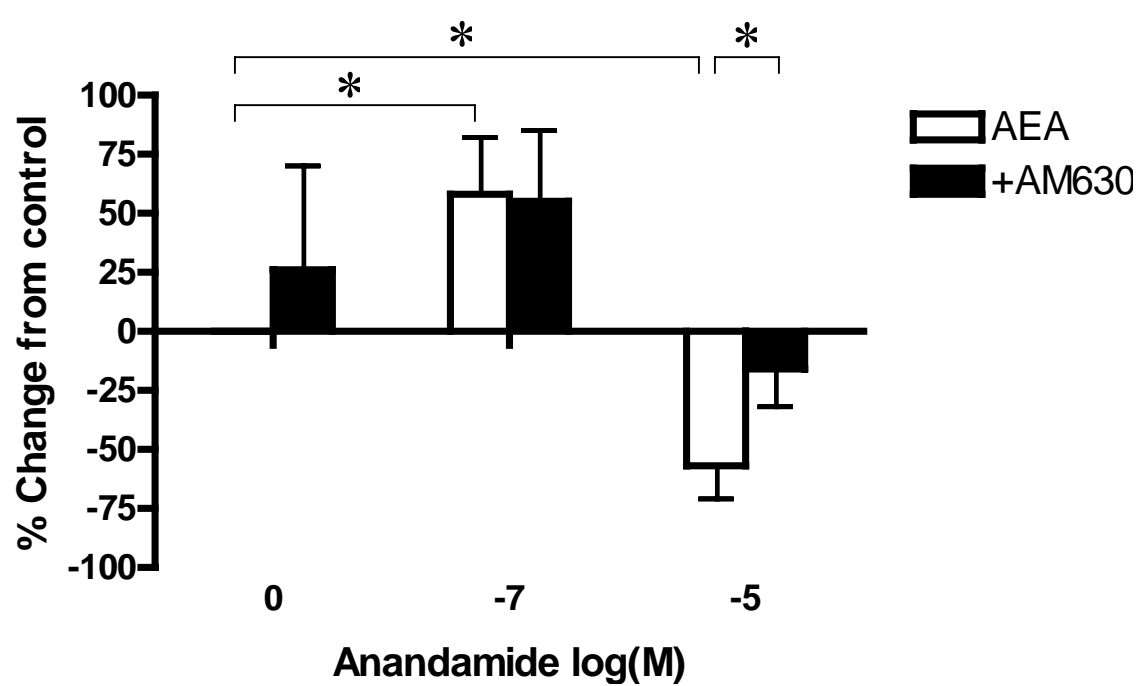


Figure 9. Anandamide dose response curve for ganglia survival with CB2 antagonist AM630 in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide had a biphasic effect on ganglionic survival, increasing survival at low concentration (0.1uM) and decreasing survival at high concentration (10uM). The effect of anandamide on decreasing survival was significantly inhibited by AM630 (1uM). Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 15 animals for control and 6 animals for AM630. \* =  $P < 0.05$ .





promoting and decreasing effects of anandamide on ganglia survival were not inhibited by TRPV1 antagonist capsazepine (1 $\mu$ M). The TRPV1 antagonist alone also had no effect on the cultured ganglia survival. These data suggest that the TRPV1 receptor may not play a role in anandamide-mediated ganglia survival (Figure 10). Taken together, these data suggest that anandamide promotes ganglia survival at low concentration (0.1 $\mu$ M) by interaction with CB1 receptors and decreases ganglia survival at high concentration by interaction with CB2 receptors.

### **Enteric glia involved in anandamide mediated ganglionic survival**

The enteric ganglia included enteric neurons and enteric glia. Next, we examined the effect of anandamide on the ganglion survival in absence of enteric glia cells. We used cytosine arabinoside (araC) to inhibit rapidly dividing cells and to reduce most of the enteric glia cells. After 7 days in culture with araC treatment, the average number of ganglia was  $4.83 \pm 1.97$  in the control group (Figure not shown). The results with anandamide treatment under these conditions are shown in Figure 11. Anandamide, at low concentrations (1nM-0.1 $\mu$ M), had no effect on ganglionic survival in presence of araC. So in the absence of enteric glia cells, anandamide lost its ability to promote ganglia survival, suggesting this effect involved the glia cells. At higher concentration 10 $\mu$ M, anandamide inhibited the ganglia survival by 63% just as it did in the absence of araC. AM251 did not reverse this inhibition, suggesting that the inhibition by anandamide was not mediated by CB1 receptor. AM251 alone had no effect on the cultured ganglia survival (Figure 12).

Next we want to know whether CB2 receptor still play a role in ganglia death in the

Figure 10. Anandamide dose response curve for ganglia survival with TRPV1 antagonist capsazepine in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the TRPV1 receptor antagonist, capsazepine (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Both promoting and decreasing survival were not inhibited by TRPV1 antagonist capsazepine. The TRPV antagonist alone also had no effect on the cultured ganglia survival. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 15 animals for control and 3 animals for capsazepine group. \* =  $P < 0.05$ .

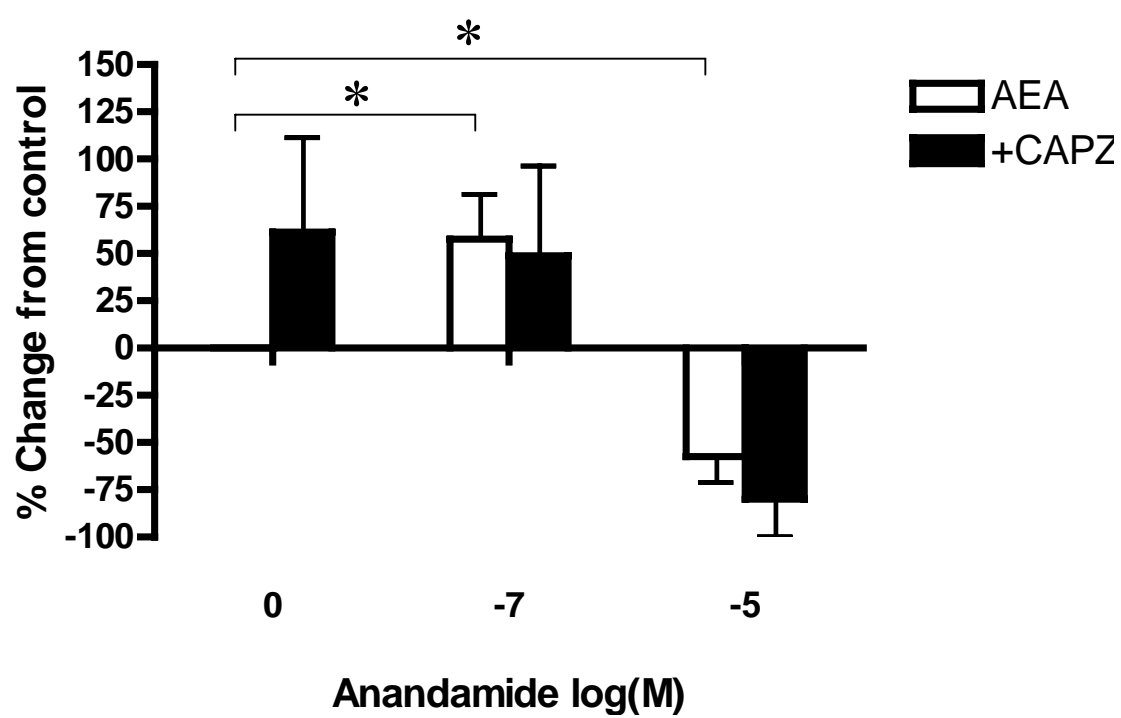


Figure 11. Anandamide dose response curve for ganglia survival in presence of araC

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide, at low concentrations (1nM-0.1uM), had no effect on ganglionic survival in presence of araC. At high concentration (10uM), anandamide inhibited the ganglia survival by 63%. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .

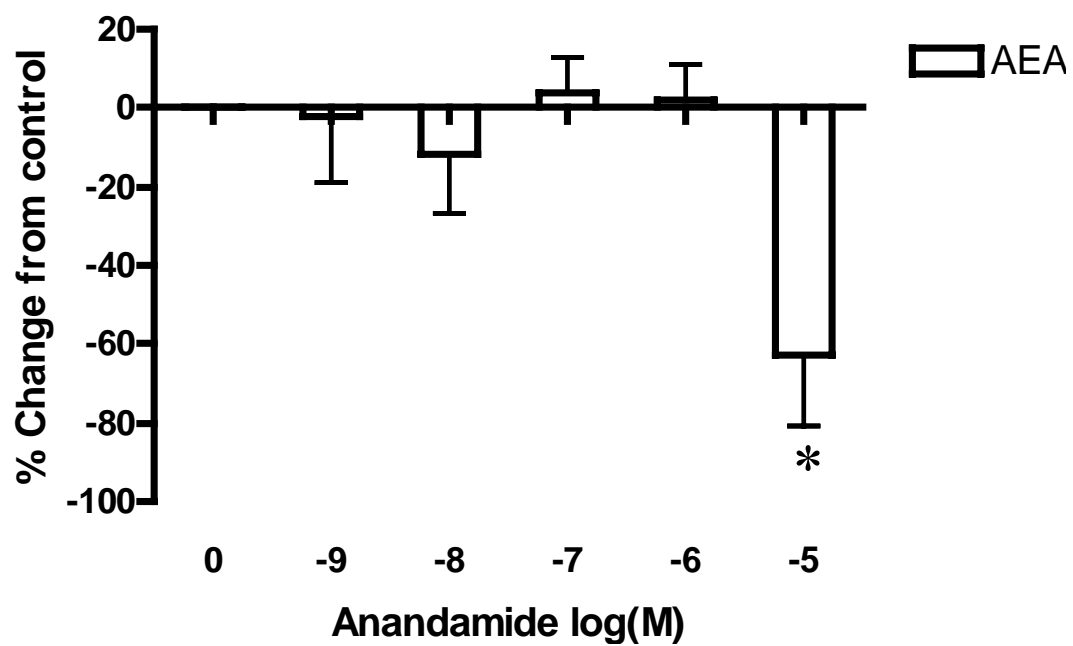
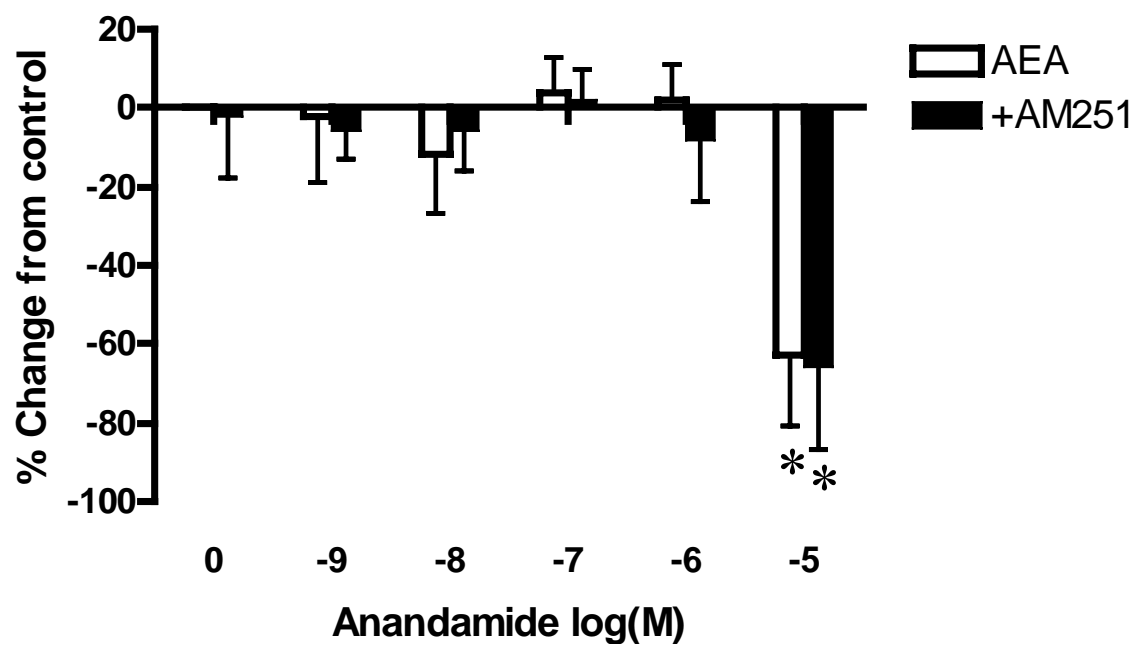


Figure 12. Anandamide dose response curve for ganglia survival with CB1 antagonist in presence of araC

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (10uM) inhibited the ganglia survival by 63%, which was not reversed by AM251. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .



absence of glia cells. As shown in Figure 13, again after reducing glia cells, anandamide, at low concentrations (1nM-0.1uM), had no effect on ganglionic survival and inhibited the ganglia survival at higher concentration (10uM). AM630 (1uM) reduced the level of inhibition of growth by high concentrations of andamide (10 uM) but this did not achieve significance, suggesting that CB2 receptors in both neurons and glia cells could participate in high concentration anandamide induced ganglia death. The CB2 antagonist alone had no effect on the cultured ganglia survival.

Taken together, these data suggested that enteric glia cells were involved in anandamide mediated ganglion survival. Anandamide may act on CB1 receptors in enteric glia to promote ganglia survival and on CB2 receptors in both enteric neurons and glia to inhibit survival.

### **Effect of anandamide on neuronal survival**

Next we want to know whether anandamide affected the enteric neuronal survival. We examined the number of neurons in each ganglia and the total number of neurons in each well. After 7 days in cultures, the number of neurons in each ganglion is  $19.11 \pm 1.44$  (Figure 14) and the number of total neurons in each well is  $116.71 \pm 14.76$  (Figure 15) in control group. Anandamide (1nM-10uM) did not change the number of neurons/ganglion significantly in presence of enteric glia (Figure 16), however, similar to the effect on ganglia survival, anandamide tended to increase the survival of total neurons per well at low concentrations (1nM-0.1uM) and decrease it at higher concentrations (1uM-10uM) in the presence of enteric glia. Maximal survival (66% increase in number of total neurons) occurred at 0.1uM (Figure 17). This data suggest anandamide could increase neuronal



Figure 13. Anandamide dose response curve for ganglia survival with CB2 antagonist AM630 in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide inhibited the ganglia survival at higher concentration (10uM) and AM630 was not able to block this inhibition significantly in the presence of araC. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 4 animals. \* =  $P < 0.05$ .

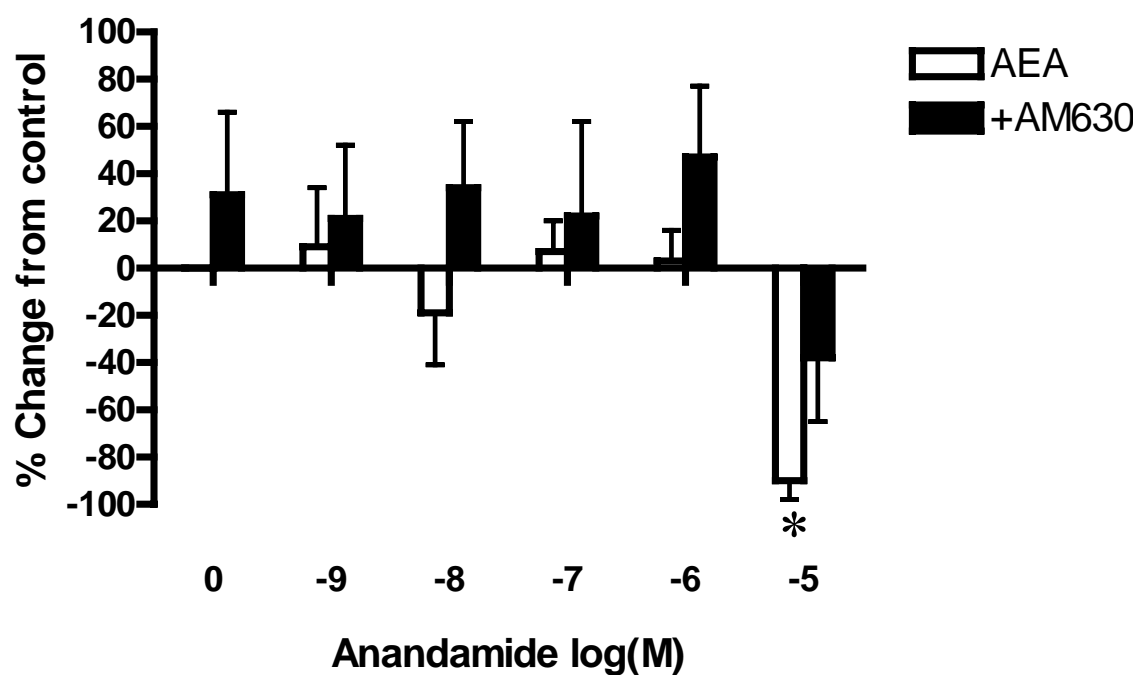


Figure 14. Effect of CB1 antagonist on neurons in ganglia in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with the CB1 receptor antagonist, AM251 (1 $\mu$ M) in DMEM0 medium for 4 days. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. CB1 antagonist AM251 (1 $\mu$ M) alone had no effect on the number of neurons/ganglion in the absence of araC. Neuronal survival is expressed as the number of neurons per ganglia. Values are means $\pm$ SEM of 9 animals.

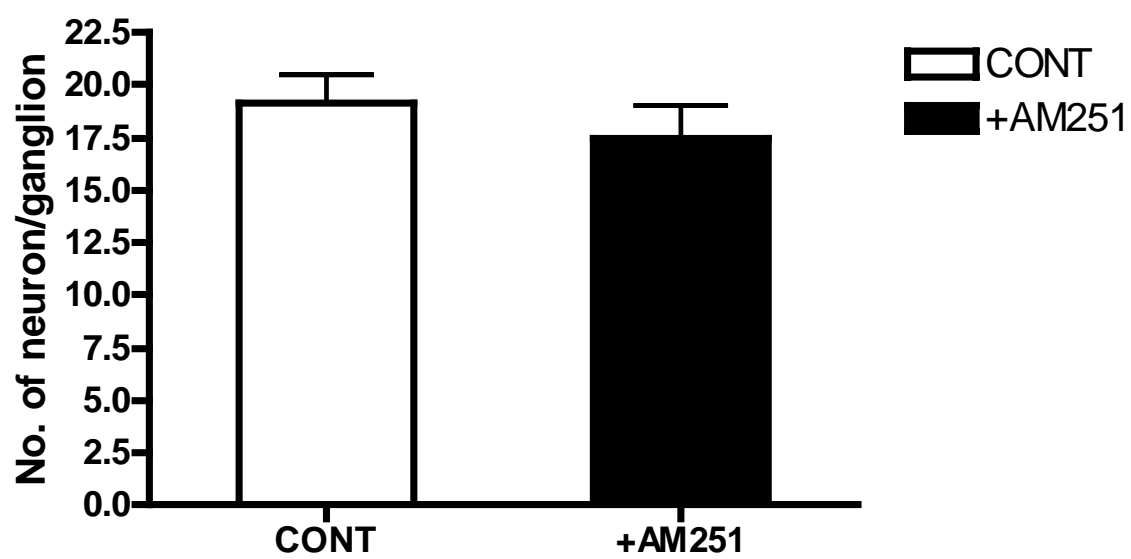


Figure 15. Effect of CB1 antagonist on total neurons per well in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with the CB1 receptor antagonist, AM251 (1 $\mu$ M) in DMEM0 medium for 4 days. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. CB1 antagonist AM251 (1 $\mu$ M) alone had no effect on the number of total neurons per well in the absence of araC. Neuronal survival is expressed as the number of neurons per ganglia. Values are means $\pm$ SEM of 9 animals.

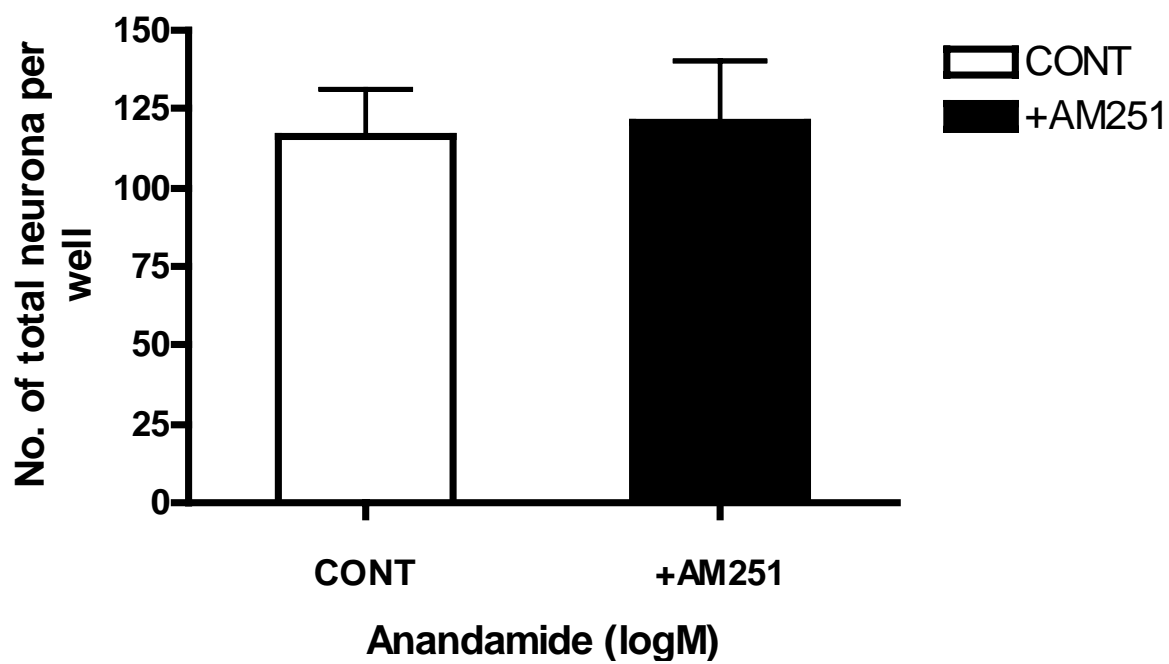


Figure 16. Neurons in ganglia dose response to anandamide in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. In the absence of araC, anandamide (1nM-10uM) did not change the number of neurons/ganglion significantly. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals.

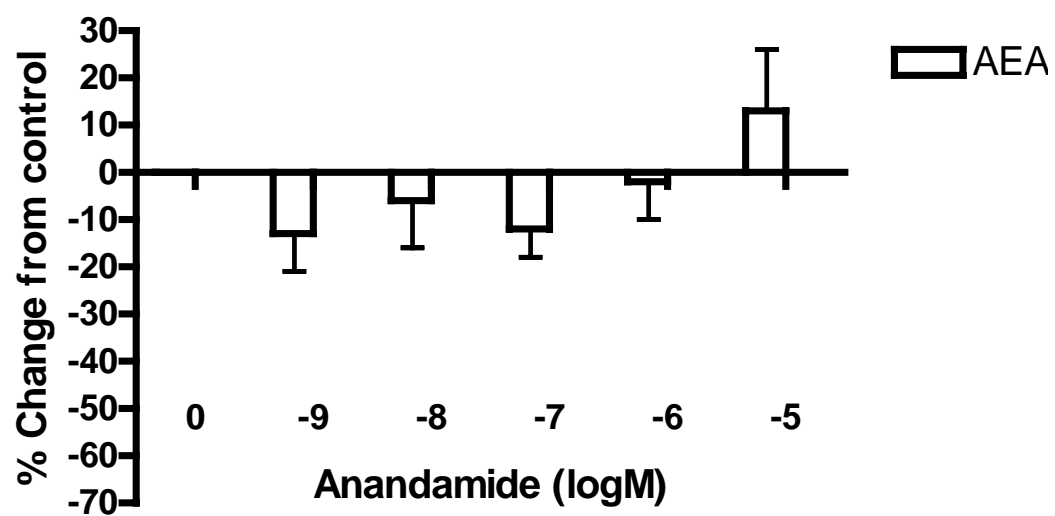
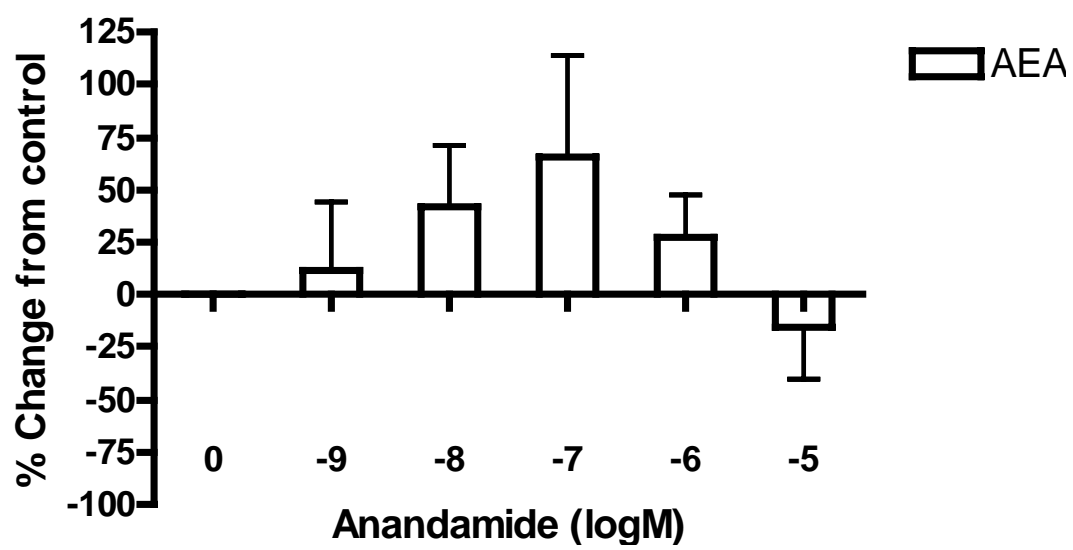




Figure 17. The number of total neurons per well dose response to anandamide in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide tended to increase the survival of total neurons per well at low concentrations (1nM-0.1uM) and decrease it at higher concentrations (1uM-10uM) in the absence of araC. Maximal survival (66% increase in number of total neurons) occurred at 0.1uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals.



survival through promoting the ganglionic survival, which is mediated by CB1 receptors. AM251 (1 $\mu$ M) alone had no effect on the number of neurons/ganglion (Figure 14) and the number of total neurons/well (Figure 15), suggesting endogenous cannabinoids may not be involved in neuronal survival through the CB1 receptor. Treatment with anandamide and AM251 together did not change the number of neurons/ganglion either (Figure 18). However, AM251 (1 $\mu$ M) tended to inhibit the effect of anandamide on total neuronal survival (Figure 19). This effect may also have been the result of the effect of AM251 on ganglion survival.

Treatment with anandamide (0.1 $\mu$ M) and AM630 together, significantly increased the neuronal survival in ganglia by 35%. AM630 alone also tended to increase neuronal survival in ganglia (Figure 20). In addition, AM630 increased total neuronal survival per well significantly when added alone or in combination with anandamide (10 $\mu$ M) (Figure 21). These data suggested that blockade of CB2 receptor may increase neuronal survival in the presence of glia and suggests that activation of CB2 receptors inhibits neuronal survival in the presence of glia.

### **Inhibition of neuronal survival by anandamide in absence of enteric glia**

As described earlier, we reduced the enteric glia cells by araC to distinguish the direct role of anandamide on neuronal survival. Low concentrations of anandamide (1nM-0.1 $\mu$ M) decreased the number of neurons/ganglion by 15-20% in the presence of araC (Figure 22). At higher concentrations, anandamide also (1nM-10 $\mu$ M) decreased the number of total neurons per well by 13-66% in the presence of araC (Figure 23). These data suggested that anandamide inhibited neuronal survival when enteric glia cells were

Figure 18. Neurons in ganglia dose response to anandamide with CB1 antagonist in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. In the absence of araC, anandamide (1nM-10uM) alone or treated with AM251 together did not change the number of neurons/ganglion significantly. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals.

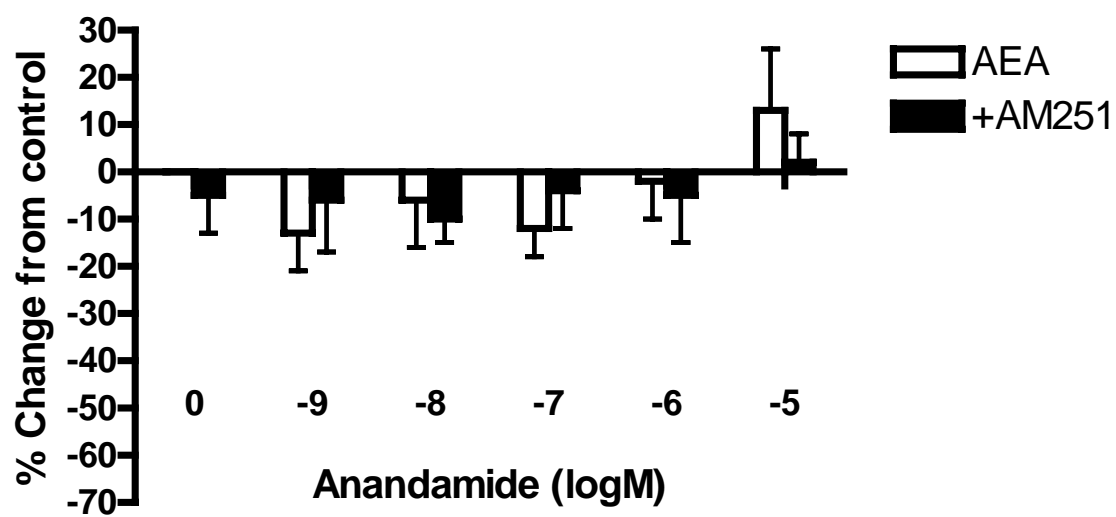


Figure 19. The Number of total neurons per well dose response to anandamide with CB1 antagonist in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide tended to increase the survival of total neurons per well at low concentrations (1nM-0.1uM) and decrease it at higher concentrations (1uM-10uM) in the absence of araC. Maximal survival (66% increase in number of total neurons) occurred at 0.1uM. AM251 (1uM) tended to inhibit the effect of anandamide at low concentrations. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 9 animals.

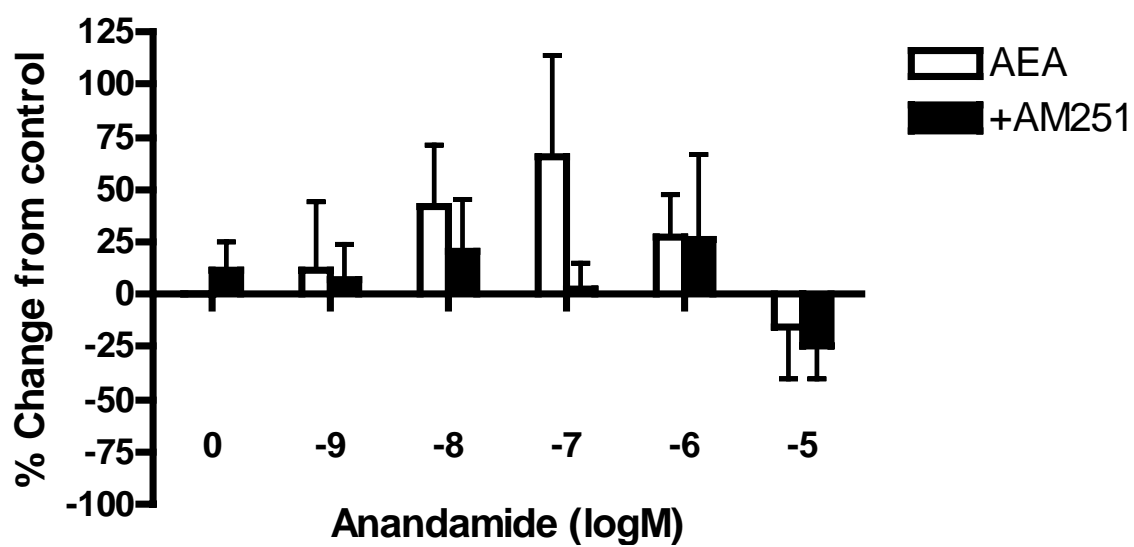


Figure 20. Neurons in ganglia dose response to anandamide with CB2 antagonist AM630 in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Treatment anandamide (0.1uM) and AM630 together, AM630 significantly promote the neuronal survival in ganglia by 35%. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 15 animals for control and 6 animals for AM630. \* =  $P < 0.05$ .



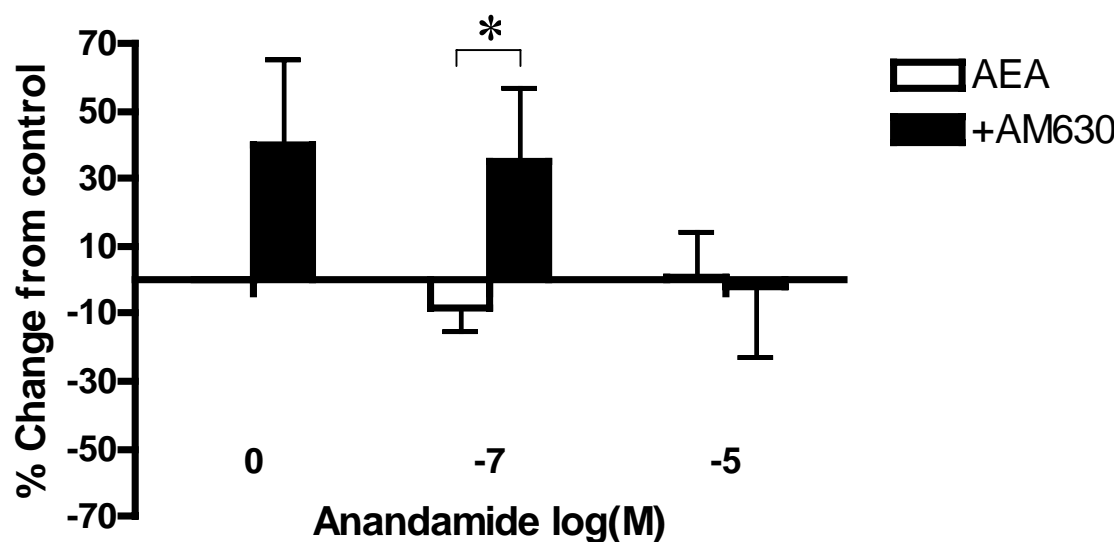


Figure 21. The Number of total neurons per well dose response to anandamide with CB2 antagonist AM630 in absence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. AM630 promoted the total neuronal survival per well significantly when treated with anandamide (10uM) together. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 15 animals for control and 6 animals for AM630 group. \* =  $P < 0.05$ .

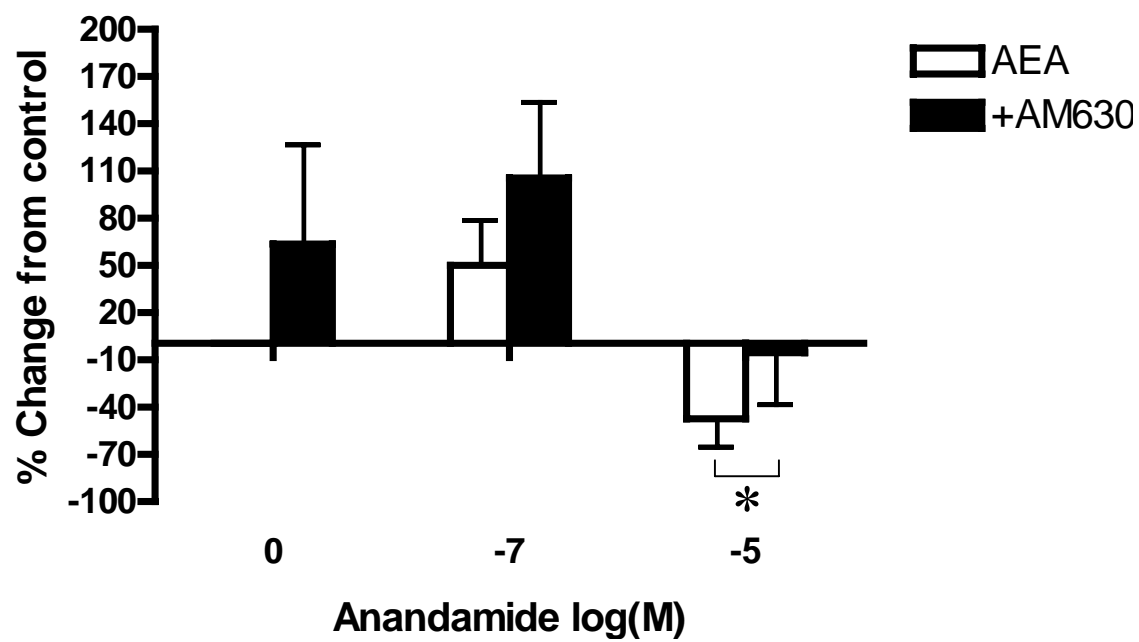


Figure 22. Neurons in ganglia dose response to anandamide in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist AM251 (1uM) on day 3. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (1nM-0.1uM) decreased the number of neurons/ganglion by 15-20% in the presence of araC. Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .

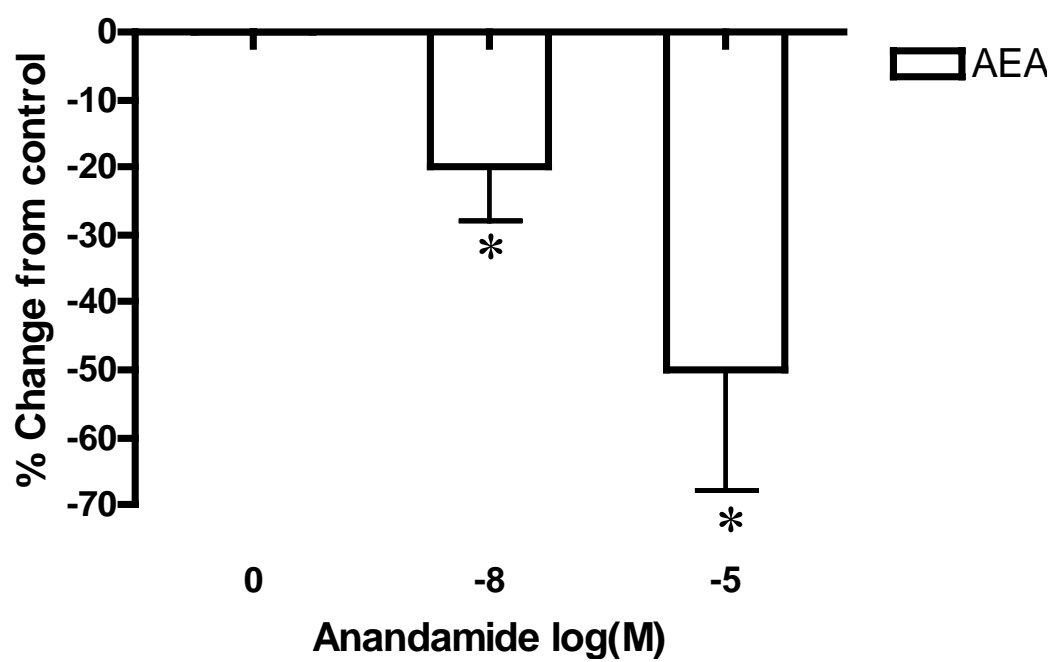
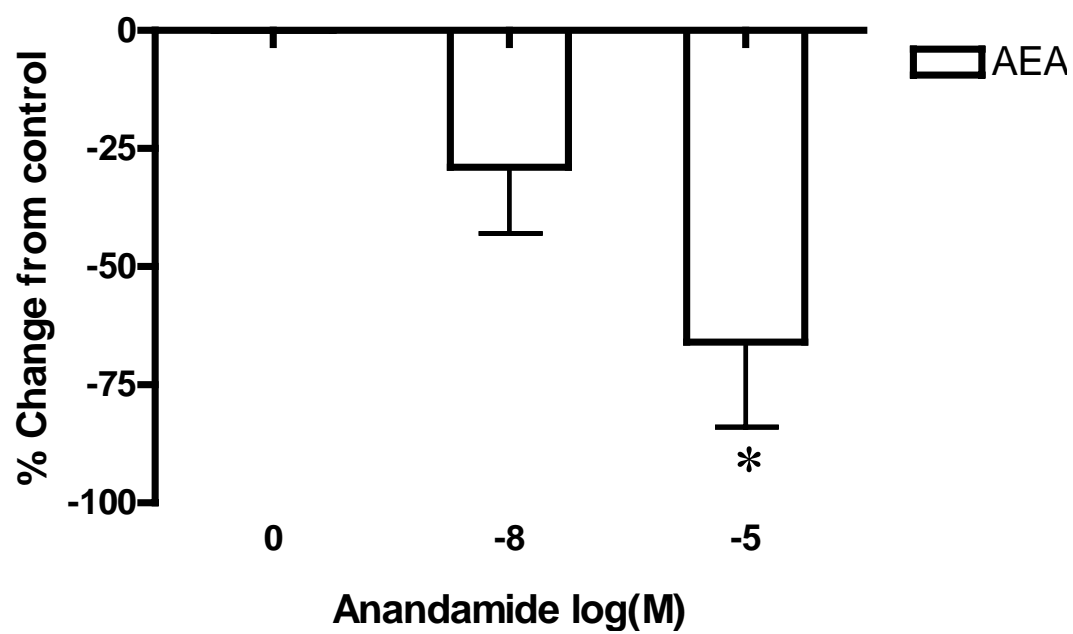


Figure 23. The Number of total neurons per well dose response to anandamide in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (1nM-10uM) decreased the number of total neurons per well by 13-66% in the presence of araC. Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .



not present in the cultures. This is very different from the condition in the presence of glia (i.e., absence of araC,) where anandamide had a biphasic effect on the number of total neurons, increasing the number of neurons at low concentrations and decreasing the total number of neurons at higher concentrations (see Figure 17).

The decrease in the number of neurons/ganglion was partially reversed by AM251 (1uM) (Figure 24 and 25), which suggested that inhibition of anandamide on the neuronal survival was partially mediated by CB1 receptors. In addition, AM251 (1uM) tended to inhibit the effect of anandamide on the number of total neurons/well at low concentrations (1nM-0.1uM) although not significantly, and had no significant effect at the highest concentration of anandamide (10uM). Again, AM251 alone had no effect on the neuronal survival in the presence of araC (Figure 25).

Next, we wanted to know whether this inhibition by anandamide also involved CB2 receptors. The results showed that again, anandamide (1nM-0.1uM) decreased the number of neurons/ganglion by 17-30% in the presence of araC and this was not significantly blocked by AM630 (1uM) although there was a tendency to reduce the effect of anandamide by AM630. AM630 alone had no significant effect on the number of neurons/ganglion in the presence of araC (Figure 26). AM630 (1uM) was also not able to significantly inhibit the effect of anandamide on the number of total neurons per well in the presence of araC (Figure 27). Although the effects of AM630 did not achieve statistical significance, they did reverse the effects of anandamide at all but the highest concentration. These data suggested that CB2 receptors are likely to be involved in anandamide mediated neuronal death in absence of enteric glia.

Taken together, anandamide decreased neuronal survival without enteric glia cells, which



Figure 24. Neurons in ganglia dose response to anandamide with CB1 antagonist in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist AM251 (1uM) on day 3. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (1nM-0.1uM) decreased the number of neurons/ganglion by 15-20% in the presence of araC. This effect was partially reversed by CB1 antagonist, AM251 (1uM). Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .

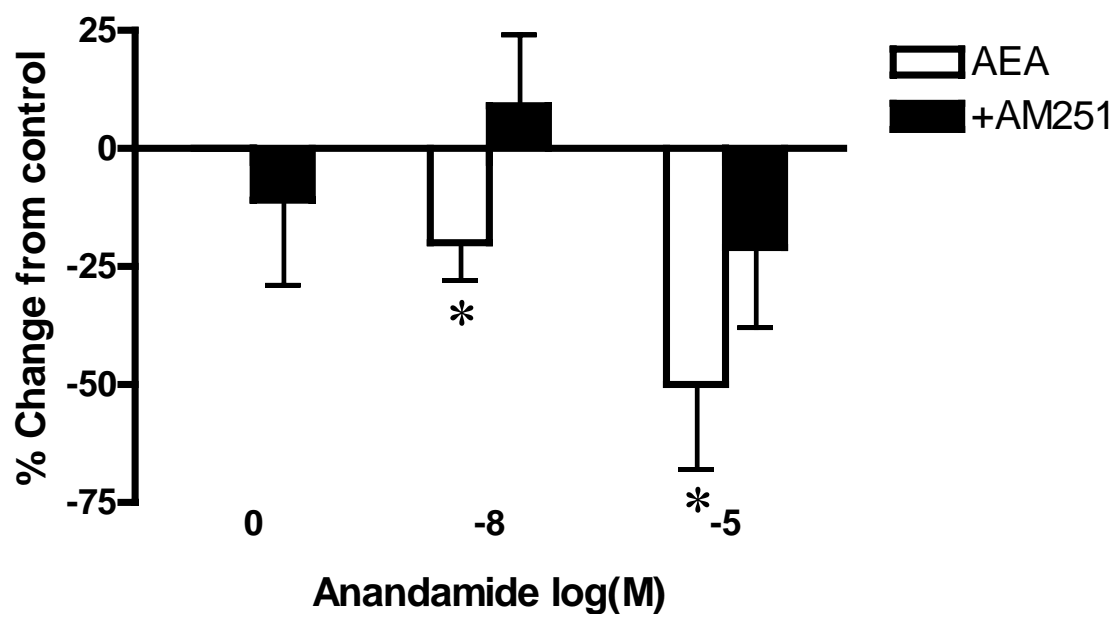


Figure 25. The Number of total neurons per well dose response to anandamide with CB1 antagonist in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB1 receptor antagonist, AM251 (1uM) on day 3. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (1nM-10uM) decreased the number of total neurons per well by 13-66% in the presence of araC, which was partially blocked by AM251. AM251 did not significantly change the effect of anandamide at 10uM. Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 7 animals. \* =  $P < 0.05$ .

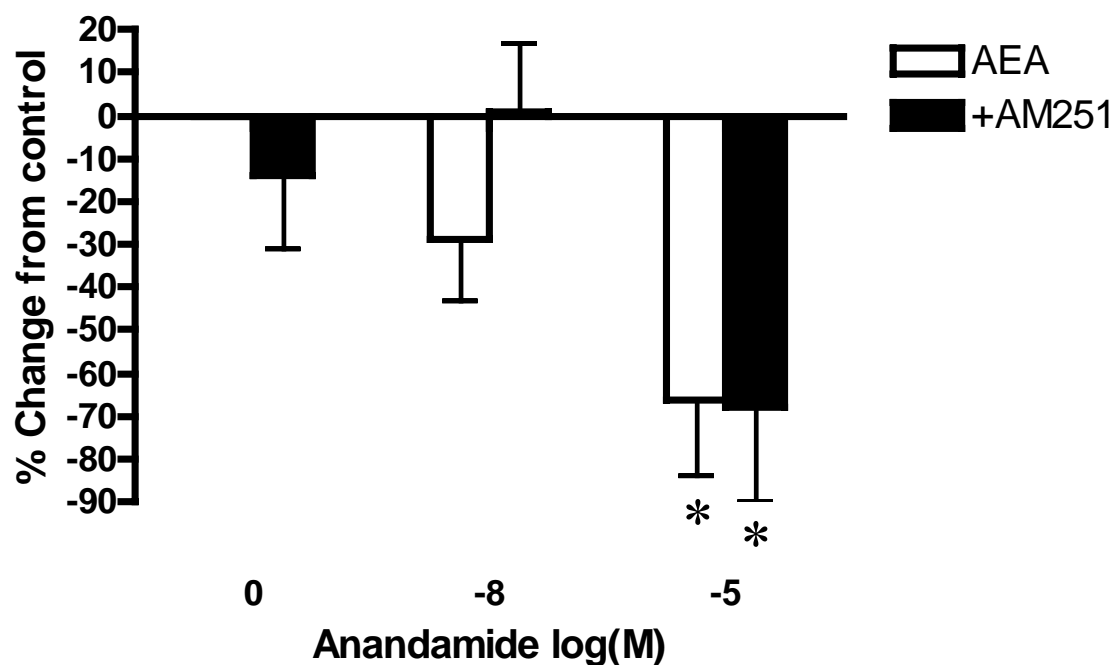


Figure 26. Neurons in ganglia dose response to anandamide with CB2 antagonist AM630 in presence of araC.

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of neurons in ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Anandamide (1nM-0.1uM) decreased the number of neurons/ganglion by 17-30% in the presence of araC. This was partially blocked by AM630 (1uM). Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 4 animals. \* =  $P < 0.05$ .

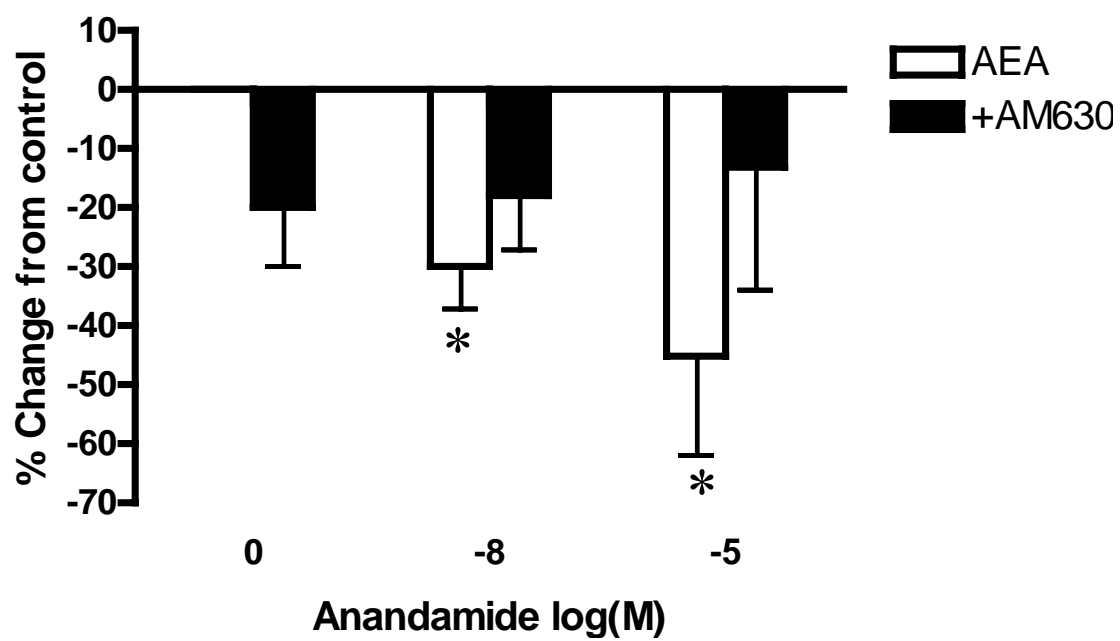
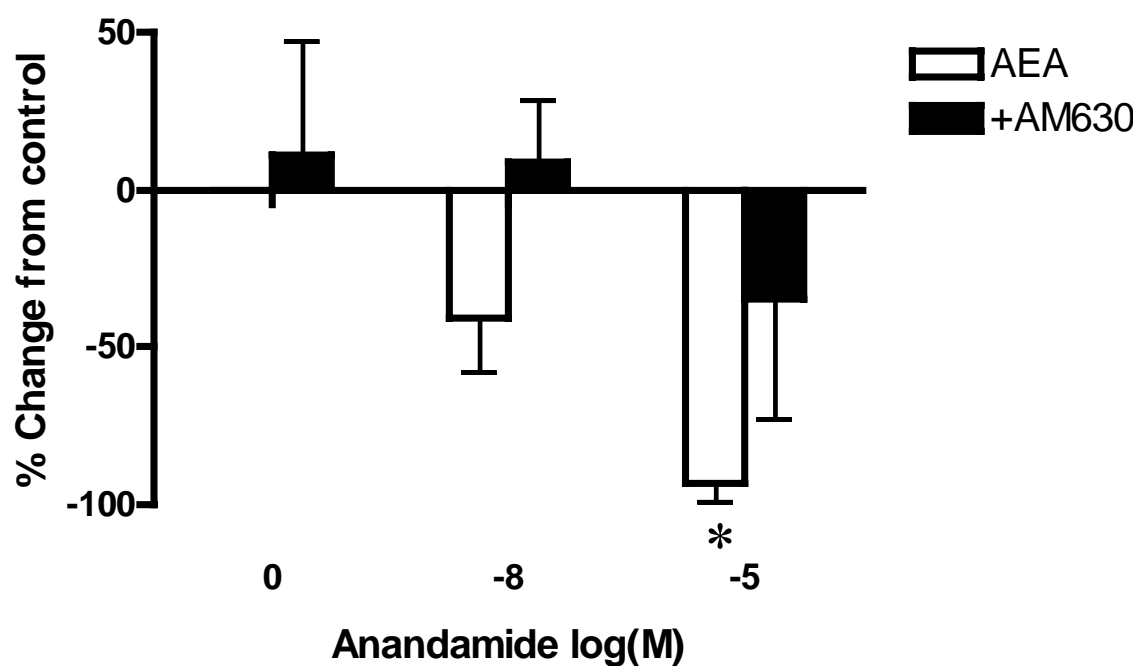


Figure 27. The Number of total neurons per well dose response to anandamide with CB2 antagonist AM630 in presence of araC

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium with cytosine arabinoside (araC; 10uM) for 3 days and then treated with anandamide (1nM-10uM) in DMEM0 medium with araC for 4 days. Some cultures were treated additionally with the CB2 receptor antagonist, AM630 (1uM) on day 3. The number of total neurons per well was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. AM630 (1uM) partially inhibit the effect of anandamide on neuronal death in the presence of araC. Data was shown in doses 10nM and 10uM. Neuronal survival is expressed as percentage change from control. Values are means $\pm$ SEM of 4 animals. \* =  $P < 0.05$ .





could be mediated by CB1 and CB2 receptors. The inhibition by lowest levels of anandamide is most sensitive to the CB antagonists whereas the inhibition induced by the highest level of anandamide is less sensitive. This data also demonstrates that the effect of anandamide is highly dependent on whether glial are present or absent from the cultures and suggests that glial may respond to cannabinoids and alter the overall effect of anandamide. We examined this in later studies of this thesis project.

### **Inhibition of enteric neuronal cell survival by anandamide**

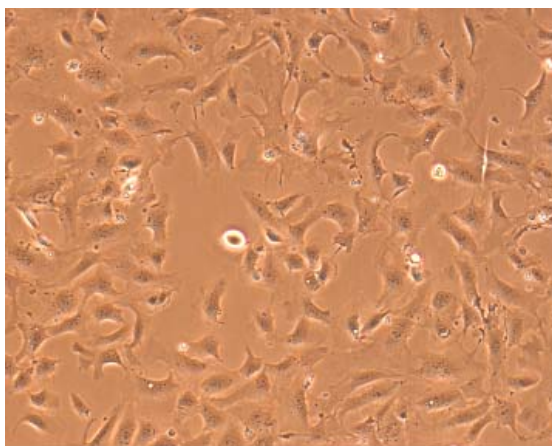
To confirm the direct inhibitory effect of anandamide on neuronal survival, we examined the newly developed immortal fetal enteric neuronal cell line (IM-FEN). Phase contrast images confirmed that the enteric cell line proliferated at 33 degree and differentiated to form neurites at 39 degree (Figure 28). Consistent with the production of neurites, most of the cells expressed the neuronal marker PGP9.5 (Figure 29); this is consistent with the report by Dr. S. Srinivasan's group at Emory University who supplied the cell line to us. In addition, this cell line was able to form ganglion-like structures and these structures further connected to the complicate networks.

Anandamide treatment at 39 degree had no effect on the number of total cells (Data not shown), however, it decreased neuronal cell percentage in a concentration-dependent manner (11-45% decrease in survival at 1nM-10uM) (Figure 30). While this is consistent with the effect of anandamide to inhibit neuronal survival in primary cultures as described above, the effect in the cell line could be due to the neuronal cell death or differentiation to non-neuronal cells or both.

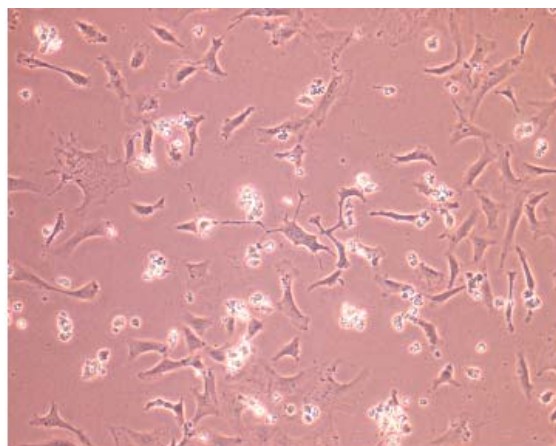
To partly address this question, we treated the cells with anandamide at 33 degree to

Figure 28. Phase contrast images of enteric cell line cultured at 33 and 39 degree.

Cells was proliferated at 33 degree, and after cultured at 39 degree for 7 days they began to differentiate with neurites.



33°C



39°C-with neurites

Figure 29. Neuronal marker PGP9.5 expression in enteric neuronal cell line (IM-FEN).

The cells were cultured in 33 degree for 2-3 days and then grown at 39 degree for 7 days.

Immunofluorescence staining was performed with anti-PGP9.5 antibody and image was shown in right panel. Most of cells expressed neuronal marker PGP9.5. Negative control was shown in left panel.

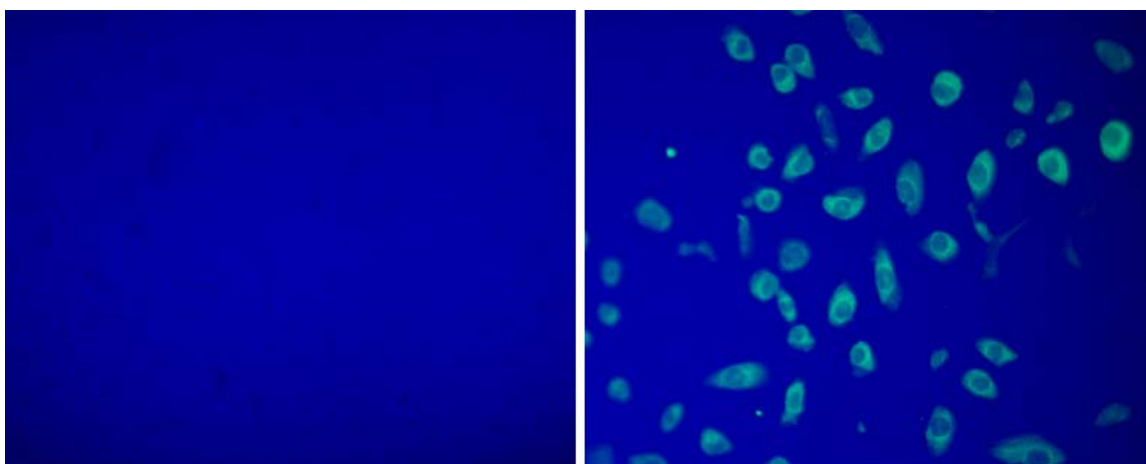
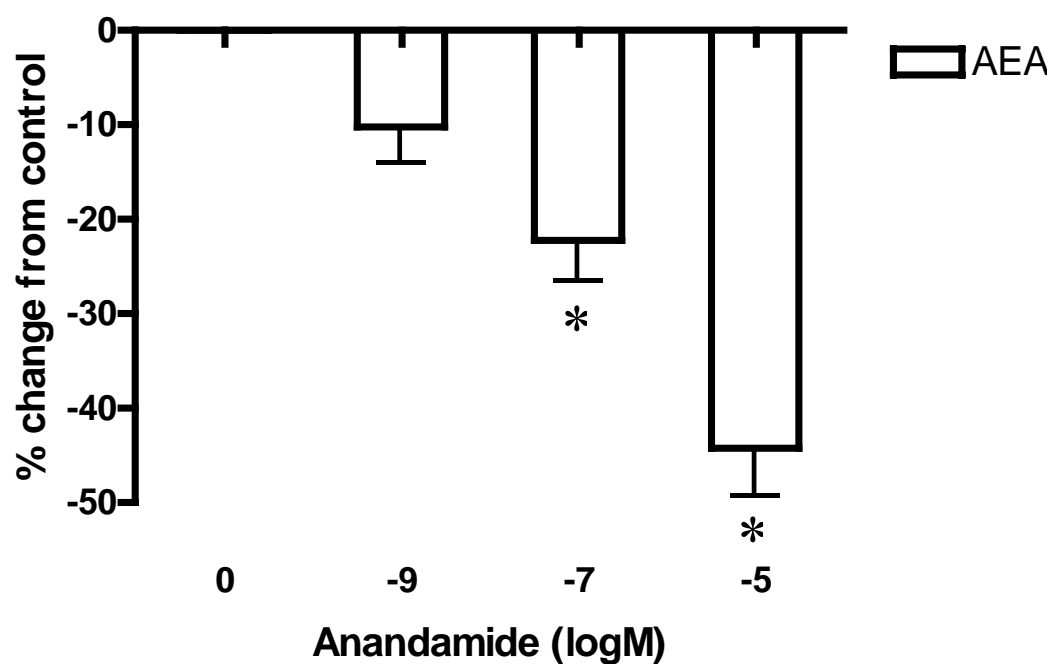


Figure 30. Anandamide dose response curve for enteric neuronal cell line at 39 degree.

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree for 2-3 days and then move to 39 degree treated with anandamide (1nM-10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. The effect of anandamide on neuronal survival was measured by counting cells immunostained with anti-PGP9.5 antibody. Anandamide treatment at 39 degree decreased neuronal cells percentage in a concentration-dependent manner (11-45% decrease in survival at 1nM-10uM). The number of total cells had no significant change among groups (Data not shown). Neuronal survival is expressed as percentage change of PGP9.5-positive cells in total cells. Values are mean  $\pm$ SEM of 4 experiments. \* =  $P < 0.05$ .



eliminate the effect of anandamide on cell differentiation at 39 degree. Anandamide also decreased neuronal survival in a concentration-dependent manner at 33 degree (10-22% decrease in survival at 1nM-10uM) (Figure 31). This further support the idea that anandamide could inhibit neuronal survival in this enteric neuronal cell line.

### **Effect of anandamide on cocultures of enteric cell line and astrocytes**

Next we want to know whether co-culture enteric cell line with glia could reverse anandamide-decreased neuronal survival. This question was based on the findings in primary cultures where the presence of glia altered the effect of anandamide. Getting enough of primary enteric glia takes a very long time (months are required to obtain a few coverslips) because these cells divide very slowly. To address this question in some manner, we used a commercially available astrocyte cell line, which was isolated from mice brain. Astrocytes were chosen because the enteric glial cells are most closely related to astrocytes. At the time of these studies, enteric glial cells were not available commercially. Unfortunately, this cell line was not able to inhibit anandamide-induced neuronal cell death (Figure 32). This lack of effect is likely due to the fact that the cells were not truly enteric glial cells and therefore neither supports or rejects our hypothesis relative to the role of glial cells in the response to anandamide.

Taken together with previous data, it illustrated that anandamide may act on CB1 receptors in enteric glia to promote ganglia survival and on CB2 receptors in enteric glia to inhibit survival. Anandamide also inhibited neuronal survival in absence of enteric glia which could be mediated by CB1 and CB2 receptors and inhibited the enteric neuronal



Figure 31. Anandamide dose response curve for enteric neuronal cell line at 33 degree.

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree treated with anandamide (1nM-10uM) in DMEM/F12 medium for 2-3 days and then moved to 39 degree without anandamide treatment for 7 days. The medium was changed once on day 3. The effect of anandamide on neuronal survival was measured by counting cells immunostained with PGP9.5. Anandamide decreased neuronal survival in a concentration-dependent manner at 33 degree. (10-22% decrease in survival at 1nM-10uM). Neuronal survival is expressed as percentage change of PGP9.5-positive cells in total cells. Values are mean  $\pm$ SEM of 3 experiments. \* =  $P < 0.05$ .

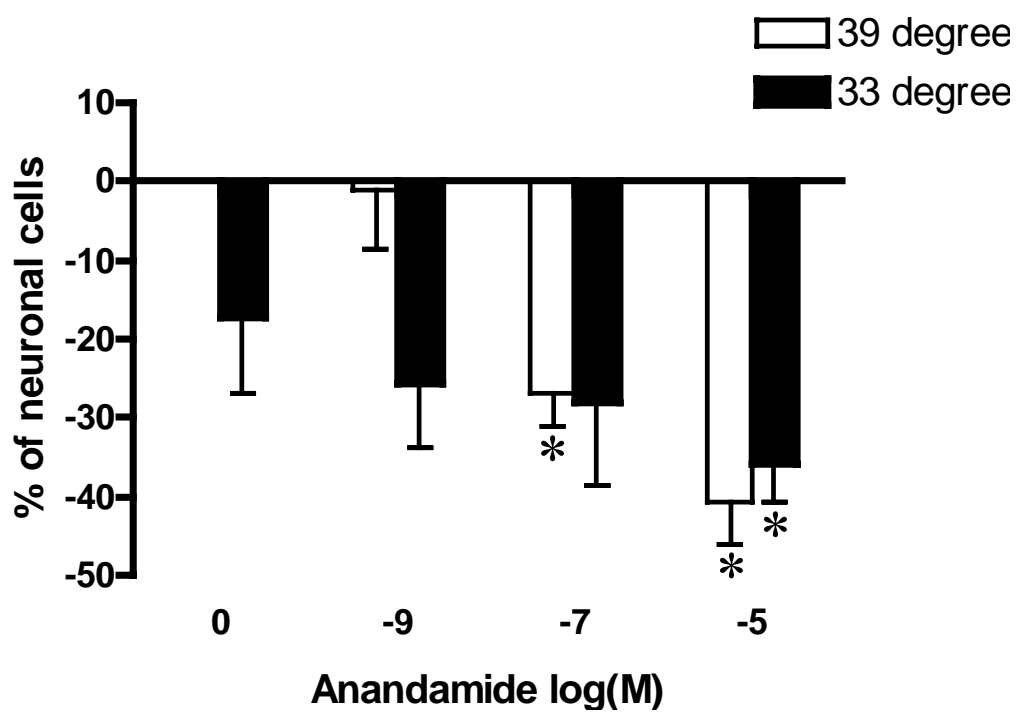
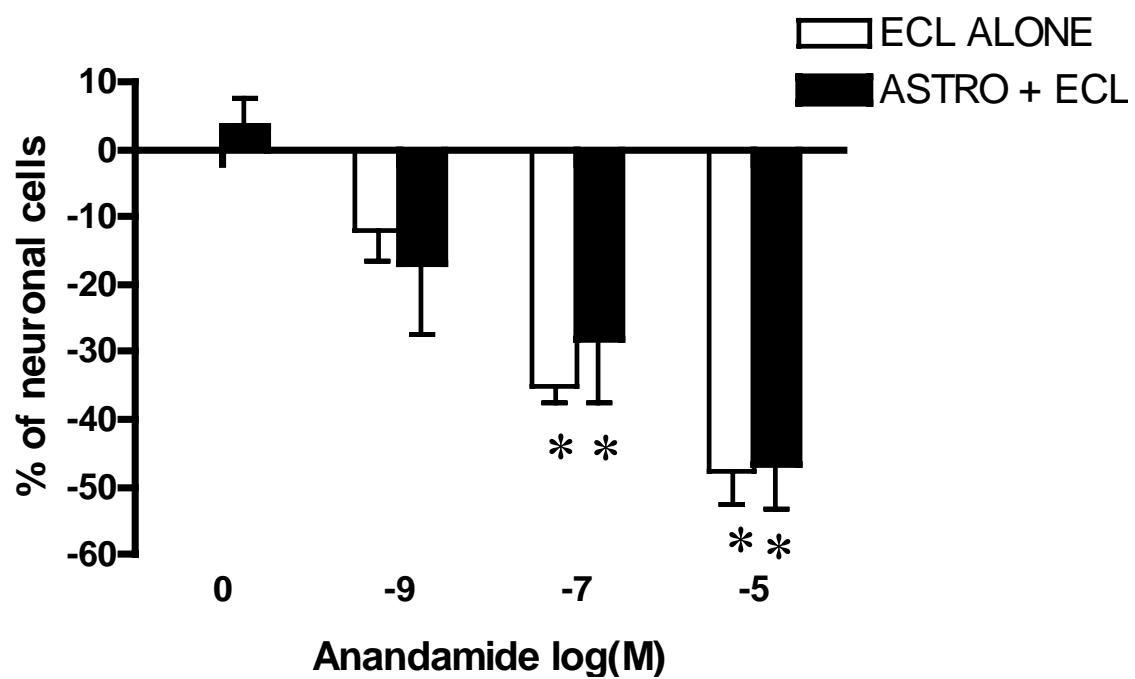


Figure 32. Anandamide dose response curve for enteric neuronal cell line cocultured with astrocyte cell line at 39 degree.

Enteric neuronal cell line (IM-FEN) was co-cultured with astrocyte cell line at 33 degree in DMEM/F12 medium for 2-3 days and then moved to 39 degree treated with anandamide (1nM-10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. The effect of anandamide on neuronal survival was measured by counting cells immunostained with anti-PGP9.5 antibody. The astrocyte cell line was not able to inhibit anandamide-induced neuronal cell death. Neuronal survival is expressed as percentage change of PGP9.5-positive cells in total cells. Values are mean  $\pm$ SEM of 3 experiments. \* =  $P < 0.05$ .



cell line in both 33 and 39 degrees.

### **CB1 and CB2 expression in primary cultures and enteric neuronal cell line**

Immunofluorescence staining showed that CB1 receptors are not only expressed in enteric neurons but also in enteric glia cells in primary cultures. However, enteric glia CB1 expression is lower than in enteric neurons. Interestingly, enteric glia cells with concentrated cell bodies had strong S100 and CB1 staining and when cell bodies spread out, the staining density became lower (Figure 33).

CB2 receptors are also expressed in both enteric neurons and glia cells in primary cultures. There are similar staining densities between neurons and glia. CB2 expression was evenly distributed in the glia cell body (Figure 34).

Both CB1 and CB2 receptors were expressed in enteric neuronal cell line, which were supported by immunofluorescence staining (Figure 35) and western blot results (Figure 36). The protein extractions from spleen tissue of guinea pig and mice were used as a positive control in western blot for CB2 antibody. (Data not shown)

### **Anandamide activated downstream signaling pathways in ganglia**

Next, we want to know which downstream signaling pathway(s) was involved in anandamide mediated ganglia survival in the presence of glial (i.e., absence of araC). We tested the MAPK pathway, PLC-beta pathway and PI3K/AKT pathway by using their inhibitors. The results are shown in Figure 37. Again, 0.1uM anandamide increased the ganglia survival in absence of araC. The PLC-beta inhibitor U73122 (1uM) alone had no significant effect on the ganglia survival (Data not shown), however, it inhibited

Figure 33. CB1 expression in primary cultures of enteric ganglia

Primary cultures of enteric ganglia were prepared from guinea pig intestine and grown in DMEM10 medium for 7 days. CB1 receptor expression was determined by immunofluorescence co-staining anti-CB1 antibody with neuronal marker PGP9.5 or glia marker S100. CB1 receptors are not only expressed in enteric neurons but also in enteric glia cells in primary cultures. Middle left: PGP9.5 (green), middle right: CB1 (red), bottom left: S100 (green), bottom right: CB1 (red). Negative controls are shown in top two. Arrows indicate examples of positive staining for enteric neurons. Arrowheads indicate examples of positive staining for enteric glia.

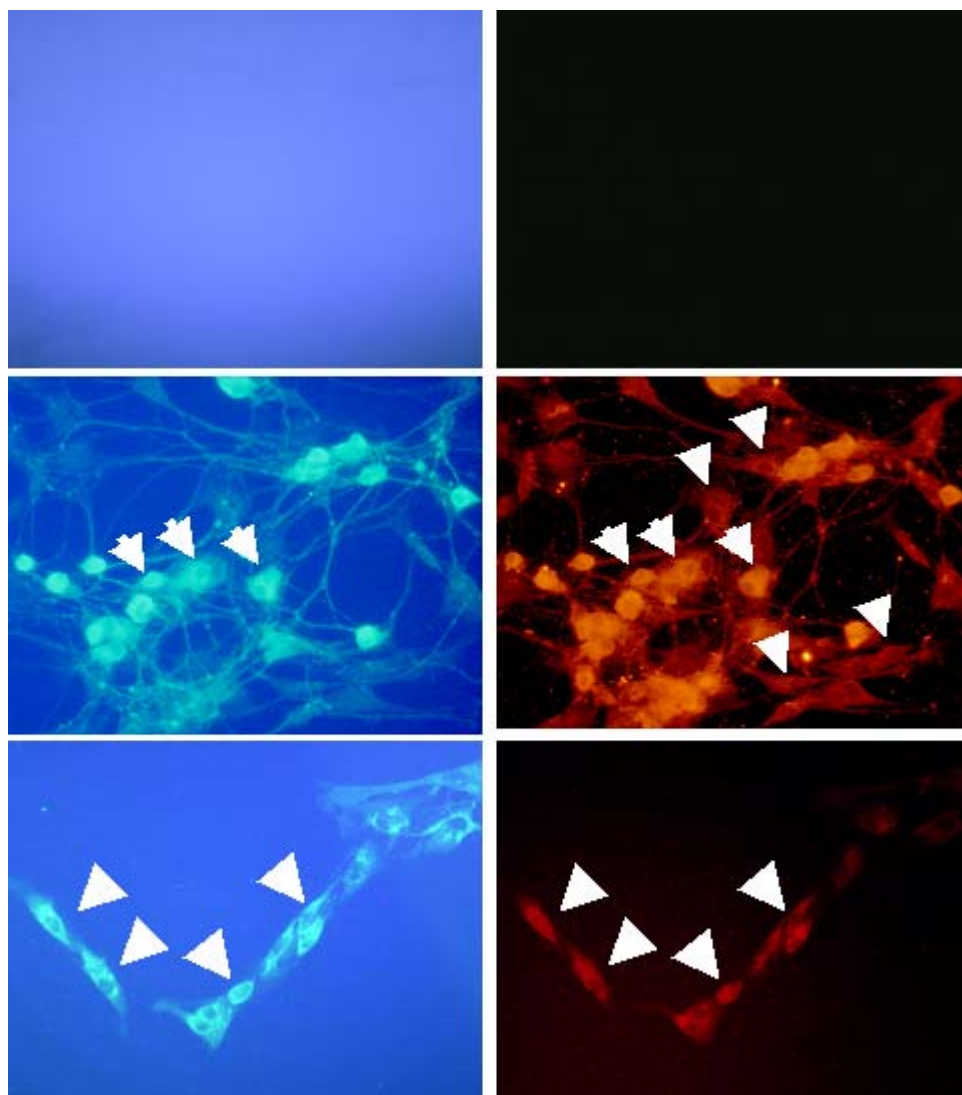


Figure 34. CB2 expression in primary cultures of enteric ganglia

Primary cultures of enteric ganglia were prepared from guinea pig intestine and grown in DMEM10 medium for 7 days. CB2 receptor expression was determined by immunofluorescence co-staining anti-CB2 antibody with neuronal marker PGP9.5 or glia marker S100. CB2 receptors are expressed both in enteric neurons and glia cells in primary cultures. Top left: PGP9.5 (green), top right: CB2 (red), bottom left: S100 (green), bottom right: CB2 (red). Arrows indicate examples of positive staining for enteric neurons. Arrowheads indicate examples of positive staining for enteric glia.



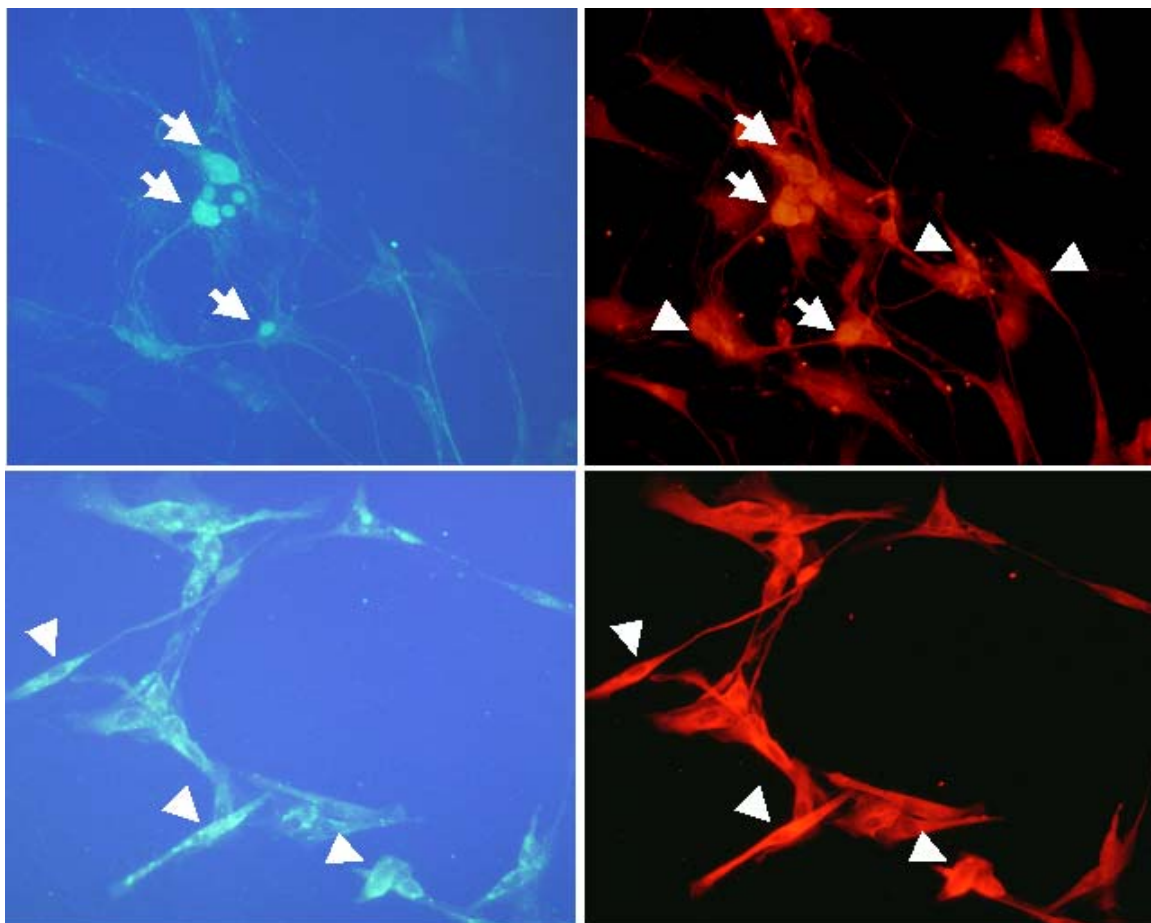
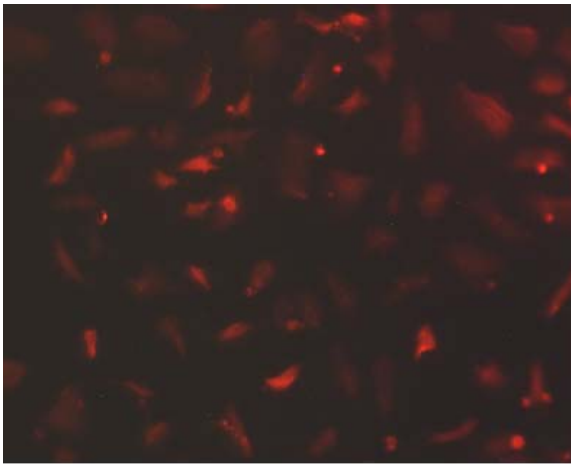
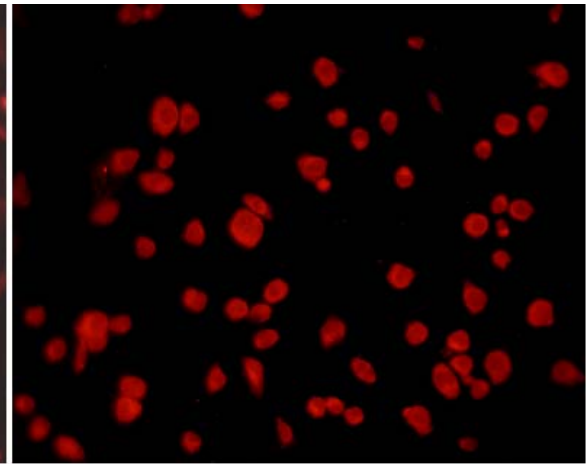


Figure 35. CB1 and CB2 receptors expression in enteric neuronal cell line (IM-FEN) by immunocytochemistry.

The cells were cultured in 33 degree for 2-3 days and then grown at 39 degree for 7 days. Immunofluorescence staining was performed with anti-CB1 or anti-CB2 antibodies and images were shown in left and right side respectively. Both CB1 and CB2 receptors were expressed in enteric neuronal cell line.



CB1



CB2

Figure 36. CB1 and CB2 receptors expression in enteric neuronal cell line (IM-FEN)

The cells were cultured in 33 degree for 2-3 days and then grown at 39 degree for 7 days.

Protein extracts were immunoblotted with either anti-CB1 or anti-CB2 antibodies and CB1 (60 KDa) and CB2 (38 KDa) bands are shown. Both CB1 and CB2 receptors were expressed in enteric neuronal cell line. Protein markers were shown in left side.

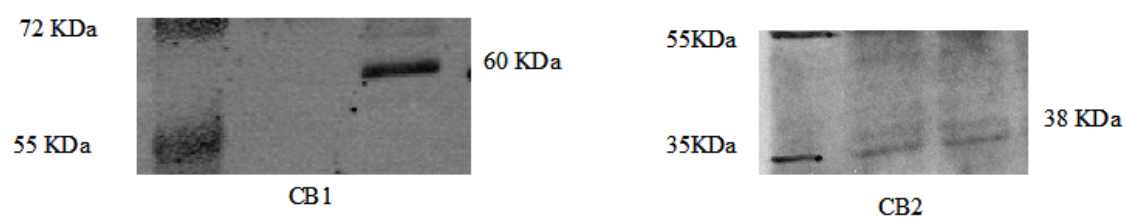
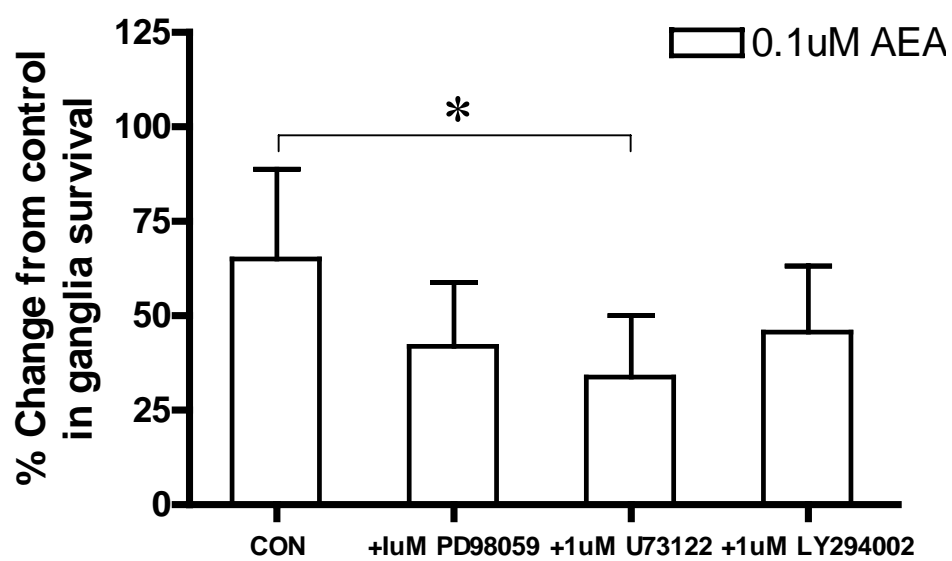


Figure 37. Anandamide mediated downstream signaling pathway

Primary cultures of enteric ganglia were prepared from guinea pig intestine. Each well was seeded with 15 ganglia grown in DMEM10 medium for 3 days and then treated with anandamide (0.1uM) in DMEM0 medium for 4 days. Some cultures were treated additionally with the MAPK inhibitor PD98059 (1uM), PLC inhibitor U73122 (1uM), and PI3K inhibitor LY-294002 (1uM) on day 3. The number of ganglia was counted on day 7 after immunostained with neuronal marker anti-PGP9.5 antibody. Again, 0.1uM anandamide increased the ganglia survival in absence of araC. PLC inhibitor U73122 (1uM) alone had no significant effect on the ganglia survival (data not shown); however, it inhibited anandamide induced ganglia survival significantly. PD98059 and LY-294002 also inhibited anandamide induced ganglia survival, although not significantly. Ganglionic survival is expressed as percentage change from control. Values are means $\pm$ SEM of 6 animals. \* =  $P < 0.05$ .



anandamide induced ganglia survival significantly, suggesting the effect of anandamide on promoting ganglia survival is mainly mediated by PLC pathway. A role of MAPK and PI3K/AKT is less well supported since antagonists of these pathways caused slight but nonsignificant inhibition of the effects of anandamide. We were not able to measure the anandamide downstream pathways by western blot in that this is a complex of enteric neurons and glia.

**Anandamide activated downstream signaling pathways in the enteric neural cell line.**

In the enteric neural cell line (IM-FEN), we tested the downstream signaling pathways involved in anandamide mediated enteric neuronal death by western blot and use of the selective antagonists. Firstly, we treated the enteric cell line with MAPK, PLC-beta, and PI3K inhibitors and examined the total cell survival. The PLC-beta inhibitor U73122 (10uM) decreased the total cells by nearly 40% (Figure 38), which made us unable to examine the effect of this compound on the neuronal percentage further. Next, we wanted to know whether the MAPK and PI3K inhibitors affect the anandamide mediated neuronal cell death. We used 10uM anandamide to inhibit the neuronal survival because the inhibition at this concentration was most significant. As shown in Figure 39, treatment with anandamide (10uM) and PD98059 (10uM) together increased anandamide-mediated inhibition up to 136%, suggesting that the MAPK pathway may protect the neuronal cells from anandamide induced death. In contrast, combination of anandamide (10uM) and LY-294002 (10uM) decreased anandamide-mediated inhibition



down to 35%, suggesting PI3K could mediate the inhibition of anandamide on neuronal percentage.

Figure 38. Effect of MAPK, PLC and PI3K inhibitors on the number of total cells at 39 degree

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree for 2-3 days and then moved to 39 degree treated with MAPK inhibitor PD98059 (10uM), PLC inhibitor U73122 (10uM), or PI3K inhibitor LY-294002 (10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. The effect of MAPK, PLC and PI3K inhibitors on the number of total enteric cell line was counted. U73122 (10uM) treatment at 39 degree decreased the number of total cells nearly 40%. Values are mean  $\pm$ SEM of 3 experiments. \* =  $P < 0.05$ .

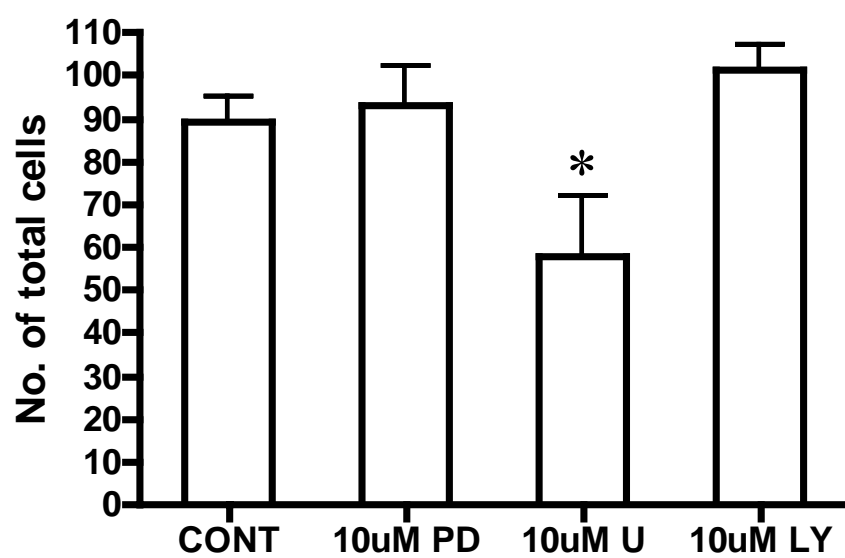
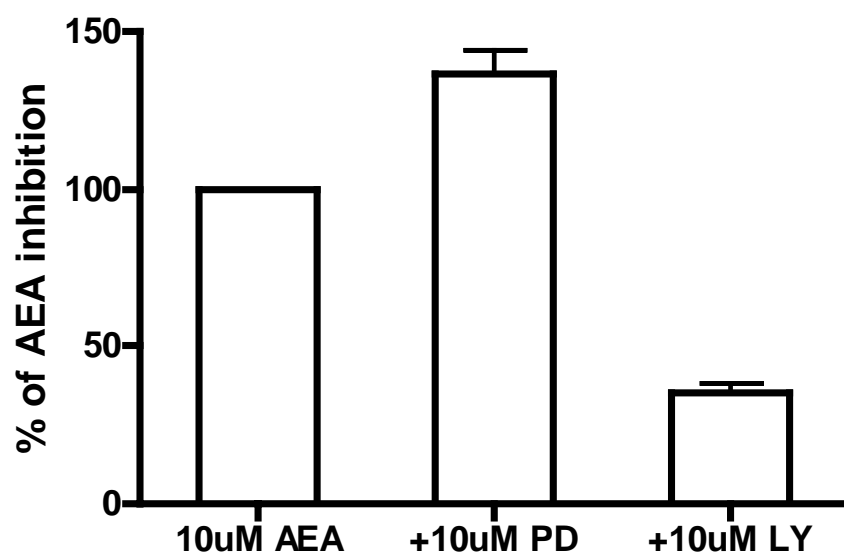


Figure 39. Effect of MAPK and PI3K inhibitors on the percentage of neuronal cells at 39 degree

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree for 2-3 days and then move to 39 degree treated with 10uM anandamide and MAPK inhibitor PD98059 (10uM) or PI3K inhibitor LY-294002 (10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. The effect on neuronal survival was measured by counting cells immunostained with anti-PGP9.5 antibody. The inhibition of 10uM anandamide in neuronal survival was normalized as 100. Treatment with anandamide (10uM) and PD98059 (10uM) together increased anandamide-mediated inhibition up to 136%, suggesting MAPK may protect the neuronal cells from anandamide induced death. Treatment with anandamide (10uM) and LY-294002 (10uM) together decreased the inhibition down to 35%, suggesting PI3K could mediate the inhibition of anandamide on neuronal percentage. Neuronal survival is expressed as percentage change of PGP9.5-positive cells in total cells. Values are mean  $\pm$ SEM of 3 experiments.



We further tested whether anandamide treatment altered the expression of p-MAPK and p-AKT by western blot. The representative bands of p-MAPK were shown in Figure 40. The quantitative results showed the expression of phospho-P44/42MAPK level was increased by 13-48% after treatment with anandamide. MAPK activation was increased most (48% increase) with 1nM anandamide treatment, and then this increase became less (36% and 13% increase, respectively) with anandamide up to 0.1uM and 10uM (Figure 41). The representative bands of p-AKT were shown in Figure 42. The quantitative results showed the expression of p-AKT level was dose-dependently increased with anandamide treatment (1nM-10uM) (Figure 43). These data support the idea mentioned earlier (see Figure 34) that MAPK and PI3K/AKT pathways were involved in anandamide-mediated inhibition of neuronal survival. We further compared the anandamide-induced change in p-MAPK and p-AKT with the anandamide induced inhibition in neuronal percentage. The curve of MAPK activation had a similar trend with the neuronal percentage inhibition and AKT activation was continually increased even when the neuronal percentage was decreased (Figure 44). The two curves crossed at around 1uM anandamide. These data suggested that the ratio of p-MAPK and p-AKT (i.e. the increasing p-AKT and the decreasing p-MAPK with increasing anandamide concentration) could be the cause of the anandamide-mediated inhibition in neuronal survival.

Figure 40. Representative western blots of P-P42/44MAPK from enteric neuronal cell line treated with anandamide.

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree for 2-3 days and then moved to 39 degree treated with anandamide (1nM-10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. The cell lysate was immunoblotted for P-P42/44MAPK. The membrane was striped and reblotted for P42/44 MAPK as control.

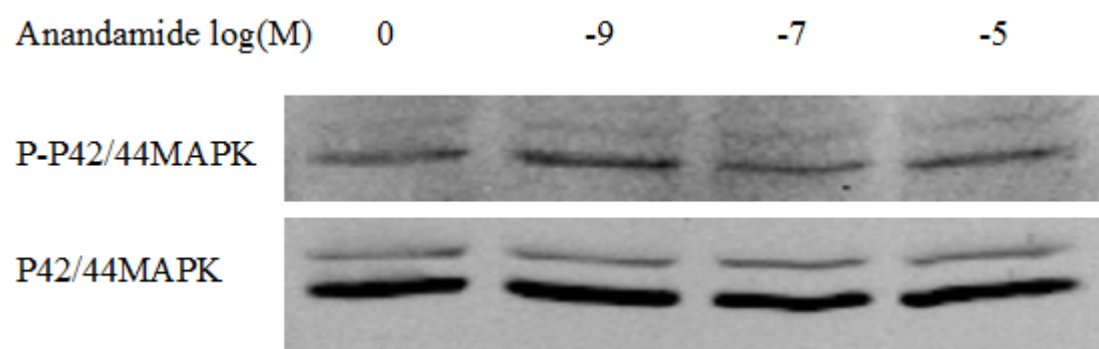


Figure 41. Quantitative analysis of phosphorylation of P42/44MAPK.

The band intensities were quantitated by odyssey software and the ratio of P-P42/44MAPK to P42/44MAPK was calculated as expression of phospho-P42/44MAPK. The expression of phospho-P44/42MAPK level was increased by 13-48% after treatment with anandamide (1nM-10uM). MAPK activation was increased by 48% with 1nM anandamide treatment, and then this increase was less (36% and 13%, respectively) with increasing anandamide concentration to 0.1uM and 10uM. Values are relative changes compared with control and expressed as mean $\pm$ SEM of 5 experiments. \* = P<0.05.



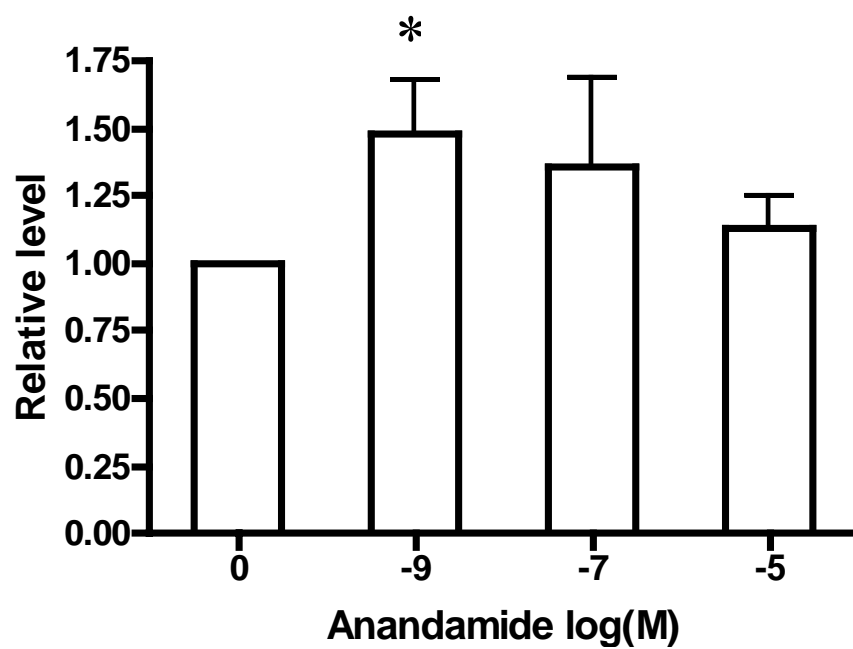


Figure 42. Representative western blots of P-AKT from enteric neuronal cell line treated with anandamide.

Enteric neuronal cell line (IM-FEN) was cultured in 33 degree for 2-3 days and then moved to 39 degree treated with anandamide (1nM-10uM) in neural basal A medium for 7 days. The medium was changed once on day 3. Protein extract was immunoblotted for P-AKT. The membrane was stripped and reblotted for total AKT as control.

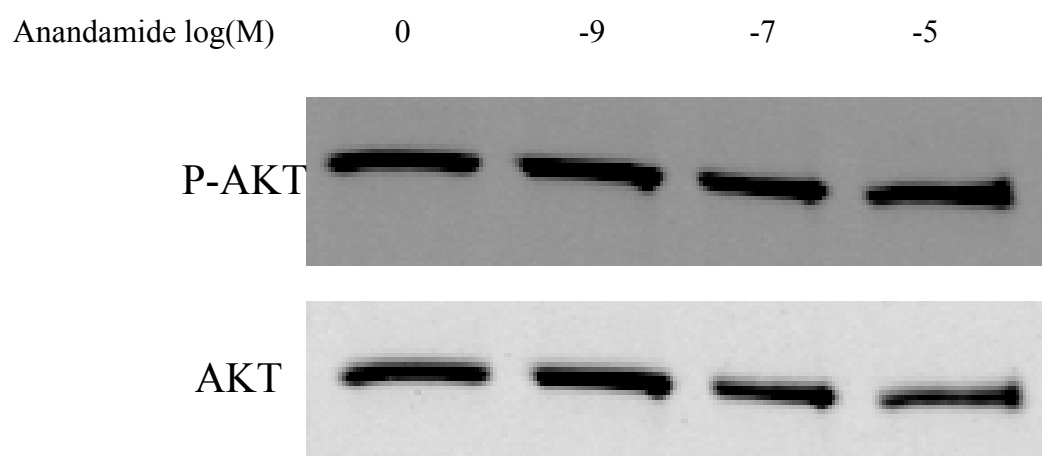


Figure 43. Quantitative analysis of P-AKT

The band intensities were quantitated by odyssey software and the ratio of P-AKT to AKT was calculated as expression of P-AKT. P-AKT level was dose-dependently increased with anandamide (1nM-10uM) treatment. Values are relative changes compared with control and expressed as mean $\pm$ SEM of 3 experiments.

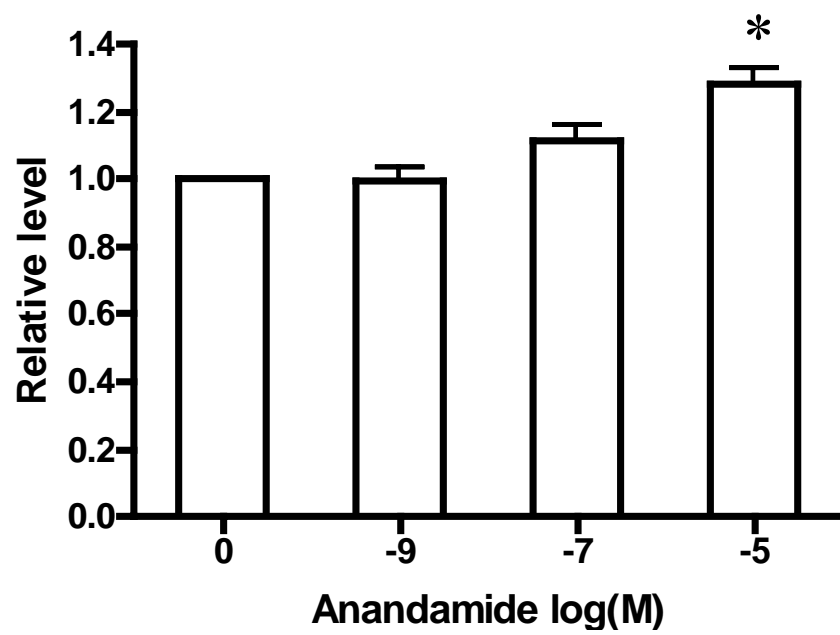
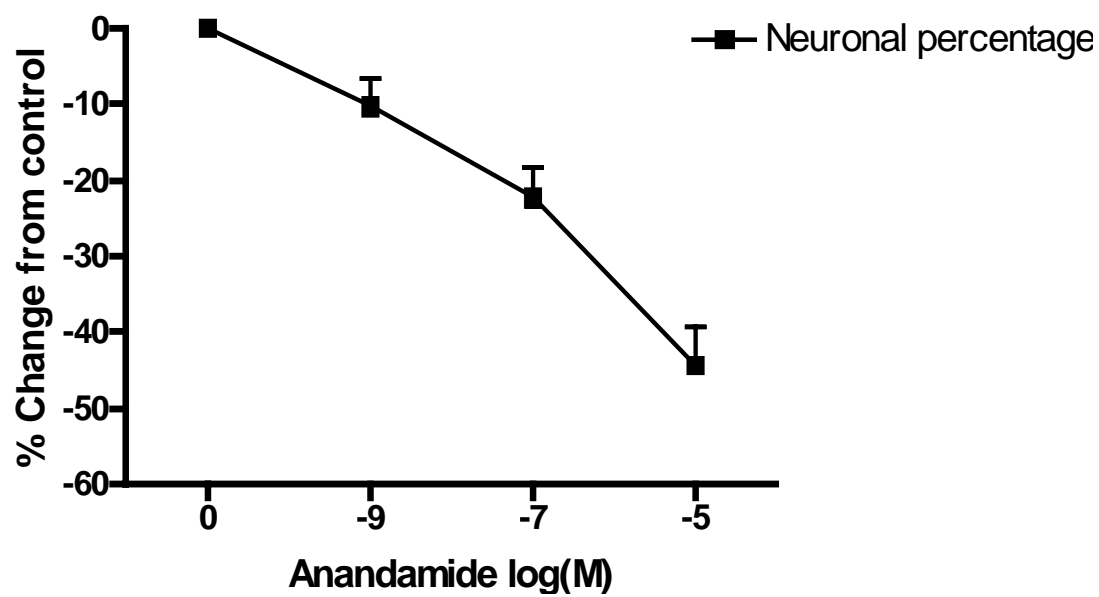
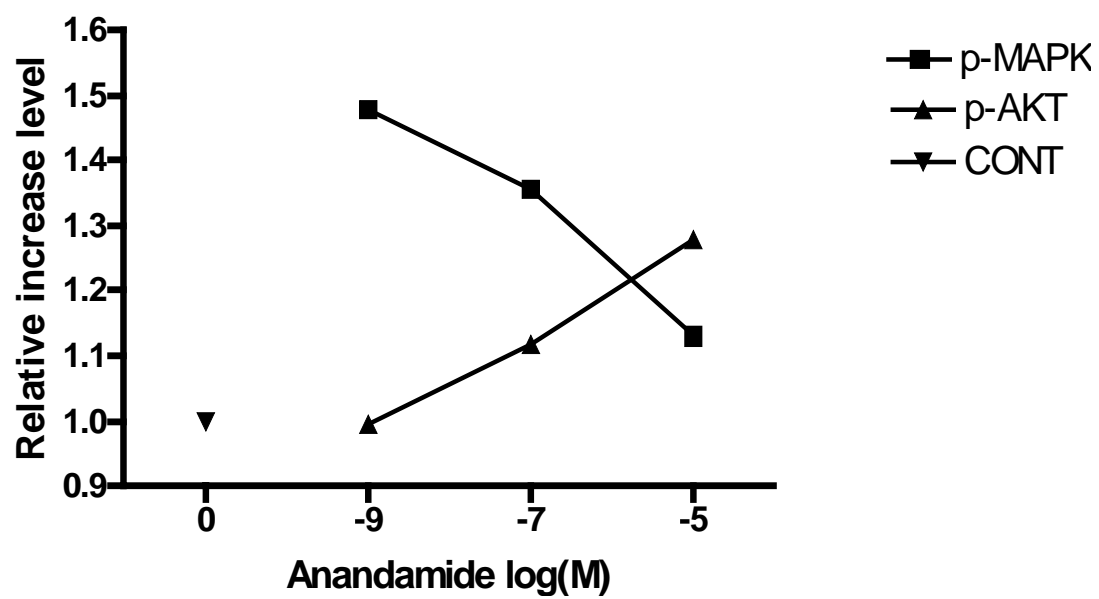


Figure 44. Comparison of MAPK and AKT signalings with the inhibition of neuronal survival by anandamide

The curve of MAPK activation had a similar trend with the neuronal percentage after anandamide treatment. AKT activation was continually increased even when the neuronal percentage was decreased. The two curves crossed at around 1 $\mu$ M anandamide. These data suggested that the ratio of p-MAPK and p-AKT could be related to anandamide-mediated inhibition on neuronal survival.







## **Discussion**

### **Expression of CB1 in myenteric neurons and glia**

The enteric ganglion is composed of both neurons and glia. The expression of CB1 receptors by enteric neurons has been well characterized (79-83,85,87,88). However, their expression by enteric glia is not clear, although there is ample evidence that they were present in astrocytes (140-144), microglial cells (91,145-152) and oligodendrocytes of the central nervous system (149). Here we show that CB1 receptors are not only expressed in enteric neurons but also in enteric glia in primary cultures of myenteric ganglia from guinea pig, which can play a relevant role in anandamide mediated ganglionic survival.

### **Anandamide promotes ganglionic and neuronal survival in presence of enteric glia**

In the present study, the effect of anandamide and cannabinoid receptors on primary cultures of myenteric ganglia and neuron survival was summarized in Table 4.

The effect of cannabinoids on neuronal survival has been studied by many groups, however, the results are controversial varying from neuroprotection (128-132) to neurotoxicity (133-134). Cannabinoid neuroprotection is usually more evident in whole-animal than in cultured-neuron models, which may result from their impact on various brain cell types (neurons, glia, vascular endothelium (153). In the present study, we have shown that anandamide increased neuronal survival through promoting the ganglionic survival in presence of glia, and that this effect is mediated by CB1 receptor.

Table 4. The effect of anandamide and cannabinoid receptors in primary cultures of myenteric ganglia and neuron survival

		Ganglia survival	Neuron/ganglia survival	Total neuron/well survival
Anandamide (AEA)	+ glia	Biphasic; Promoting ganglia survival at 1nM-0.1uM; Inhibiting ganglia survival at 10uM	No effect	Promoting total neuron survival at 1nM-0.1uM
CB1 activation	+ glia	Mediate AEA-induced promoting ganglia survival	No effect	Mediate AEA induced promoting neuron survival
CB2 activation	+ glia	Mediate 10uM AEA-induced inhibiting ganglia survival	Inhibition of neuron/ganglion survival	Mediate 10uM AEA induced inhibition of neuron survival
TRPV1	+ glia	No effect	Not tested	Not tested
Anandamide (AEA)	- glia	Inhibition of ganglia survival at 10uM	Inhibition of neuron/ganglion survival	Inhibition of total neuron survival at 10uM
CB1	- glia	No effect	Partially mediate AEA induced inhibition of neuron survival	No effect
CB2	- glia	Partially mediate 10uM AEA-induced inhibiting ganglia survival	Partially mediate AEA induced inhibition of neuron survival	Partially mediate 10uM AEA induced inhibition of neuron survival
TRPV1	- glia	Not tested	Not tested	Not tested

This clearly suggested glia could play an indispensable role in cannabinoid-mediated neuroprotection. However, the exact mechanism is still not clear. Some studies have shown that cannabinoids protect glial cells from ceramide induced apoptosis in vivo and in vitro (154) and from oxidative stress damage through CB1 receptors (155). In addition, CB1 receptor-mediated neuroprotection might result from cross-talk to the growth factor system. For example, chronic exposure to  $\Delta^9$ -THC up-regulated brain derived neurotrophic factor (BDNF) in specific brain areas in vivo (156) and BDNF is known to be involved in CB1 receptor-dependent protection against excitotoxicity (157). Thus, anandamide could protect enteric glia from death thereby maintaining the ganglia integrity and also transactivate the tyrosine kinase receptors to protect ganglionic and neuronal survival.

#### **Anandamide promotes ganglionic survival through CB1 and PLC-beta primarily**

CB1 receptors are G protein-coupled seven-transmembrane receptors (GPCR) (158). They are preferentially coupled to PTX-sensitive Gi/o proteins (33-34). Recently some studies showed they could also couple to Gq/11 proteins (144,159-160). Activation of CB1 receptors can activate PLC-beta in a PTX-sensitive manner mediated by the Gi/o  $\beta\gamma$  subunits (161-163) or PTX-insensitive manner mediated by the Gq/11 (144,159-160). In the present study, we show that anandamide mediated ganglionic survival was blocked by the PLC-beta inhibitor U73122, suggesting this effect was mediated by the PLC-beta pathway. However, whether it is Gi/o or Gq/11 mediated has not been tested yet. Context-specific activation of G proteins could partially explain the complexity of cannabinoid effect in the nervous system. In addition, it is not completely clear where activation exactly happens: enteric neurons or glia. Some studies suggest that the

cannabinoid agonist WIN 55212-2 could activate the CB1 receptor in astrocytes directly to induce  $\text{Ca}^{2+}$  elevation through activation of PLC-beta (144). Our studies also suggest that there may be a possibility that the effect of cannabinoids on ganglionic survival activates the MAPK and PI-3K/AKT pathways but to a lesser extent than the PLC-beta pathway. Using the selective antagonists of these pathways, we found a tendency to decrease ganglionic survival although this decrease did not achieve statistical significance.

### **CB2 expression in enteric neurons and glia**

The CB2 receptor was originally found in immune cells, where one of its roles being to modulate cytokine release (49). Later it was detected morphologically and functionally in neurons in central nervous system (164) and enteric nervous system (89). In the present study, we also show that CB2 receptors are expressed in enteric neurons in primary cultures of myenteric ganglia from guinea pig. In addition, we found that enteric glia expresses CB2 in this culture condition as well. This is in contrast with other studies, which did not observe CB2 receptor expression on enteric glia under normal conditions and in the LPS-treated tissues for 2 hours in whole mount preparations of the rat myenteric plexus (89). This difference could be explained in several ways. It could be that CB2 expression was inducible in enteric glia by culturing up to 7 days. In this point, enteric glia could have certain microglial properties. Since studies have shown that microglial cells in primary cultures are intrinsically activated or “primed” because of the procedures involved in transferring these cells into culture (165) and primed microglia prepared from human, rat or mouse tissue express CB2 receptors (91,146-148,166-168). It could also be that our primary cultures have the advantage that glia cells spread out and

attach to the bottom of wells instead of wrapping together with neurons and ganglia, as they would in the whole mount preparations, thus providing increased access to immunostaining and visualization of the CB2 staining. In addition, it could be a species difference in that our studies were done in guinea pig whereas the other studies were done in rat whole mounts.

Enteric glia were found in enteric ganglia, the interconnecting nerve strands of the ganglionated plexuses (14). Subsequently, enteric glia bodies and processes were clearly demonstrated within the mucosal plexus (15-17), and glial processes make close contacts with the epithelial cell layer (15,17-20). Interestingly, CB2 immunoreactivity was detected in the epithelium of colonic tissue from inflammatory bowel disease (88). This suggests a role for this receptor in inflammation, although whether it is expressed by enteric glia in mucosal plexus have not been tested.

### **Role of CB2 in myenteric ganglionic survival**

Activation of CB2 receptors has been implicated to be actively involved in the gut response to inflammation including decreasing motility (111) and inhibiting local histological damage (112). In addition, activation of CB2 receptors reduced the basal sensitivity and the TNBS colitis-induced hypersensitivity to colorectal distension in rats (115) and inhibited the endogenous immunogenic agent bradykinin response in murine mesenteric afferent nerve activity (169).

There are few data about the role of CB2 receptors in astrocytes and most studies about CB2 function in glia came from microglial cells involving an anti-inflammatory role. (For review, see 168). Studies have shown that stimulation of CB2 receptors by 2-AG increases Erk activity in monocytes and increases migration of monocytes (73,170-172).

CB2 receptor activation decreases the *in vitro* production of proinflammatory molecules in rat microglial cells (147, 173), and human microglial and THP-1 cells (174). More recently the studies show that activation of CB2 receptors by 10uM anandamide inhibits LPS/IFN $\gamma$ -induced production of IL-12 (p35/40) and IL-23 (p19/p40) through ERK1/2 and JNK pathways in microglial cells (175). Stimulation of CB2 receptors by 2-AG also increases microglial cell proliferation (176). However, in the present study we found that anandamide decreased ganglia survival at high concentration (10uM), and this effect appeared to be mediated by CB2 receptors; suggesting activation of CB2 receptors could induce ganglia death. Our data appears to be contradictory to the results mentioned above. In our primary ganglia system where enteric neurons and enteric glia coexist, neuron-glia communication is an important factor for mediating ganglia survival. This is a very different relationship and is different from the studies utilizing the microglial cell line (176). Another possible reason for the difference could be due to the different agonists used. Anandamide acts as a partial agonist while 2-AG functions as a full agonist for CB2. Moreover, the agonistic activity of 2-AG was attenuated by anandamide (177). Both anandamide (0.036 nmol/g tissue) and 2-AG (44 nmol/g tissue) are found in the gut (110), and understanding how these two endogenous agonists modulate CB2 receptors could be important for elucidating the mechanisms of endocannabinoid in gut inflammatory diseases. It is important to note too that we find different effect of anandamide depending on the concentration. It is not known whether in physiological or pathological conditions, the local concentration of anandamide could be up to the 10uM level necessary to activate CB2 receptors.

### **Role of CB2 in enteric neuronal survival**

It has been proposed that activation of CB2 receptors is neuroprotective (see review 178). CB2 receptors were up regulated or inducible after pathological neuroinflammatory insults and activation of CB2 receptors provided neuroprotection in neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). These effects have been shown to be mainly through a series of glia-dependent anti-inflammatory actions. For example, activation of CB2 receptors also reduces the release of proinflammatory factors including nitric oxide, TNF $\alpha$ , IL-1 and IL-6 in animal models of perinatal hypoxia–ischaemia (179) and Huntington's disease (180). However, a recent study has shown *in vivo* and *in vitro* that the exogenous cannabinoids delta-9-tetrahydrocannabinol and CP55940 inhibit the chemotactic response of microglia to *Acanthamoeba culbertsoni*, an opportunistic pathogen that is the causative agent of Granulomatous Amoebic Encephalitis, through activation of the CB2 receptors (181,182). In the present study, we have shown that AM630 significantly promote the neuronal survival in ganglia by 35% when treatment together with anandamide (0.1 $\mu$ M) in the presence of glia. AM630 alone tended to increase neuronal survival in ganglia as well. These data suggest that activation of CB2 receptors could decrease neuronal survival in the presence of glia. Since 0.1 $\mu$ M anandamide, would be too low to activate CB2 receptors, our data suggests there is an endogenous tone of endocannabinoids to activate CB2 receptors to decrease the neuronal survival in the primary cultures of ganglia. This may be mediated by release of endogenous 2-AG, which is a preferential agonist of the CB2 receptor. This possibility could be tested in future studies.



### **Anandamide inhibits enteric neuronal survival in absence of enteric glia**

Anandamide has also been reported to induce cell death in rat cortical neuronal cultures (134). In addition,  $\Delta 9$ -THC had a neurodegenerative effect in cultured cortical neurons as well and it appeared to involve the CB1 receptor (133,135). In the present study, we show that anandamide (1nM-0.1uM) inhibit neuronal survival in the absence of glial cells (i.e. presence of araC). This effect was partially reversed by CB1 antagonist, AM251 (1uM) and by the CB2 antagonist AM630. This suggests that the anandamide induced decrease in neuronal survival without enteric glia cells, could be mediated by both the CB1 and CB2 receptors. To confirm the direct effect of anandamide on neurons, next we looked at the newly developed immortal fetal enteric neuronal cell line (IM-FEN). Anandamide decreased the percentage of neuronal cells in a concentration-dependent manner at both 39 and 33 degree. This further supports the idea that anandamide could inhibit survival of the neuronal cells.

### **Anandamide mediated pathways in enteric neuronal cell line**

We also found that anandamide increased the expression of phospho-P44/42MAPK in the enteric neuronal cell line although the lowest concentrations of andamide caused the most increase in P-MAPK. As the concentration of anandamide increased, the stimulation of p-MAPK decreased and the percentage of neuronal survival also decreased. This suggests that anadamide may have a dual effect on MAPK and that MAPK may have a protective effect on neuronal survival. Thus increasing levels of anandamide decrease the stimulation of p-MAPK leading to decrease protection from cell death. Cannabinoids mediated MAPK activation has previously been reported in the rat cerebral frontal cortex (183), the dorsal striatum and the nucleus accumbens (NA) (184) and Neuro2a cells

(185). A role for cannabinoids-induced apoptosis involved MAPK has also been demonstrated previously in leukemic cell lines (186) and human breast cancer cells (187). The previous studies also showed that the tumour suppressor protein, P53 (135), and Calpain (134) were involved in the cannabinoids-mediated cell death in cultured cortical neurons. However, whether these were related to MAPK has not been tested yet. Our studies also demonstrated that anandamide increased levels of p-AKT and that increasing levels of anandamide led to increased levels of p-AKT. Thus, the levels of p-AKT paralleled the increased neuronal death as the concentration of anandamide increased. This pattern is the exact opposite to that described for p-MAPK. Thus, it may be that rising p-AKT levels and falling p-MAPK levels and or changes in the ratio of these signal pathways may be the cause of the neuronal cell death caused by increasing levels of anandamide. This would be an area for future investigations. We should note for completeness that we also tested the PLC-beta antagonist, U73122. This agent caused a decrease in the total number of cells (both neural and non-neural cells) in the cultures. This effect prevented us from examining the role of PLC-beta in these IM-FEN cultures and we therefore did not examine whether or not anandamide caused the production of PLC-beta in these cells. This leaves open the possibility of an additional role of this signaling pathway in mediating the effects of anandamide.

### **Role of TRPV1 in myenteric ganglionic survival**

It is well known that sensory neurons express TRPV1 receptor. The previous studies have shown that endocannabinoids stimulate intestinal sensory neurons via the TRPV1 to release SP, resulting in ileitis in rats (108). In addition, the TRPV1 antagonist capsazepine ( $10^{-5}$ M) inhibited neuropeptide release, including somatostatin, substance P,

and CGRP, from isolated rat tracheae induced by high concentrations of anandamide ( $5 \times 10^{-5} \text{M}$ ,  $10^{-4} \text{M}$ ) (188). However, TRPV1 receptors have not been found in the enteric glia cell so far. In the present study, we found that both the stimulating and inhibiting effects of anandamide on ganglionic survival were not inhibited by the TRPV1 antagonist capsazepine, suggesting TRPV1 receptor may not play a role in anandamide-mediated ganglia survival. This could be due to the suggestion that TRPV1 was not expressed in enteric glia and had little effect on enteric glia survival.

### **Formation of networks in enteric neuronal cell line**

Nerves of the enteric nervous system derive from migratory vagal neural crest cells and sacral neural crest cells, which enter the foregut at embryonic day (E) 9-9.5 in mice and reach the terminal colon by E14 to E15 (see review 189). The fetal enteric neuronal cell line (IM-FEN) was isolated from the intestines of E13 immortomice (H-2K<sup>b</sup>-tsA58 transgenic mice) fetuses using p75NTR antibody to separate out neural precursor cells (139). Previous studies have shown that bone morphogenetic protein (BMP) is necessary for neural crest cell migration and ganglion formation in the enteric nervous system in chick (190). In the present study we found that this cell line was able to form ganglion-like structures and these structures further interconnected to form complicated networks. To form this network, the cells need proliferate to 100% confluence so that they can be very close to each other. This phenomenon can be observed both in 33 and 39 degrees, suggesting they have this ability even without differentiation in 39 degree. We also observed that the surrounding cells tended to move toward to the center of ganglion-structure. This implies that there are certain signaling molecules produced by the cells that act as a chemoattractant to induce this movement. Interestingly, the cells in the core

of this structure stain positive for PGP9.5, suggesting that the chemoattractant derives from the neuronal cells, but not others. This is also supported by our studies in coculture enteric neuronal cell line with the astrocyte cell line. We found that the astrocyte cell line alone was not able to form this ganglion-like structure even though they are 100% confluent, however, the ganglion-like structures and network formed when they were cocultured together with enteric neuronal cell line. This observation suggest that this function in vitro could mimic the process of enteric nervous system development in vivo and could be a good model for studying the ganglia colonization and the mechanisms of Hirschsprung's disease. It also suggest that our studies of the effects and mechanims of action of cannabinoids in the IM-FEN cell line are a good model for the actions of cannabinoids in the studies of primary ganglia isolated from the guinea pig intestine.

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